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(54) Title: ENDOPLASMIC RETICULUM STRESS TRANSCRIPTION FACTOR		
(57) Abstract <p>To provide a factor capable of efficiently regulating expression of an endoplasmic chaperone gene, a nucleic acid encoding it or a complementary strand nucleic acid thereof, a method for regulating expression of an endoplasmic reticulum chaperone gene, and a method for expressing a foreign gene. An endoplasmic reticulum stress transcription factor capable of regulating transcription-inducing activity, wherein the transcription-inducing activity is exhibited by an element having the nucleotide sequence as shown in SEQ ID NO: 1 or an element having a nucleotide sequence resulting from substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1; a method for controlling expression of an endoplasmic reticulum chaperone, comprising expressing the factor; a method for expressing a foreign protein, comprising positively regulating expression of an endoplasmic reticulum chaperone gene by the method for controlling expression; a nucleic acid encoding activated form of ATF6, activated form of CREB-RP, suppressive form of ATF6 or suppressive form of CREB-RP, or the complementary strand thereto. It is expected to be applied to treatment or prophylaxis of cancers, arteriosclerosis, cystic fibrosis, ischemic diseases, wounds or ulcers.</p>		

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- 1 -

DESCRIPTION

ENDOPLASMIC RETICULUM STRESS TRANSCRIPTION FACTOR

5 TECHNICAL FIELD

The present invention relates to a factor capable of efficiently regulating expression of an endoplasmic reticulum chaperone gene, a nucleic acid encoding it or a complementary strand nucleic acid thereof, a method for
10 regulating expression of an endoplasmic reticulum chaperone gene, and a method for expressing a foreign gene.

BACKGROUND ART

15 Mammalian cells, like other eukaryotic cells and prokaryotic cells, have developed a number of homeostatic mechanisms to cope with to various physiological and environmental conditions that threaten their survival. Among them, the tightly regulated synthesis of heat shock
20 proteins (HSPs) is a well-known mechanism universally found in all organisms. In addition, the regulated synthesis of glucose-regulated proteins (GRPs), based on a mechanism differing from that of HSP described above, is specifically found in eukaryotic endoplasmic reticulum
25 [Lee, A.S., *Curr. Opin. Cell Biol.* 4, 267-273 (1992);

- 2 -

Morimoto, R.I. et al., *The Biology of HEAT SHOCK PROTEINS and MOLECULAR CHAPERONES*, Cold Spring Harbor Laboratory (1994)].

In mammals, eight kinds of GRPs, namely, GRP78/Bip, 5 GRP94/ERp99, ORP150/GRP170, ERp72, GRP58/ERp60/ERp61, calreticulin, protein disulfide isomerase (PDI) and FKBP13, have been identified. These GRPs are a series of molecular chaperones or folding enzymes characteristic of the endoplasmic reticulum, each of which expression is 10 induced by the accumulation of proteins that have failed to be folded or have undergone incorrect folding (hereinafter referred to as unfolded proteins) in the endoplasmic reticulum (endoplasmic reticulum stress) [Kozutsumi, Y. et al., *Nature* 332, 462-464 (1988); Lee, 15 A.S., *Trends Biochem. Sci.* 12, 20-23 (1987)], and play a very important role in the folding of nascent secretory proteins and membrane proteins in the endoplasmic reticulum. Therefore, these GRPs are hereinafter generically referred to as "endoplasmic reticulum 20 chaperones".

Expression of endoplasmic reticulum chaperones is also induced by a reagent, such as tunicamycin, which inhibits N-glycosylation of proteins, calcium ionophore A23187, which depletes calcium storage, or thapsigargin, 25 which inhibits calcium-ATPase. These reagents are

- 3 -

generally assumed to cause malfunctioning of the endoplasmic reticulum and elicit endoplasmic reticulum stress.

Induction of the above endoplasmic reticulum chaperone by endoplasmic reticulum stress is primarily regulated at the transcription level. Since the endoplasmic reticulum chaperone is not induced by heat shock stress and the promoter sequence of the endoplasmic reticulum chaperone gene contains no heat shock elements, the induction of endoplasmic reticulum chaperone is suggested based on a regulatory mechanism differing from that for the HSP induction. There has yet remain unknown, however, whether an endoplasmic reticulum stress-response is controlled by a common mechanism or by a variety of mechanisms corresponding to individual endoplasmic reticulum chaperones.

The rat GRP78 gene has already been analyzed to some extent, and it is shown that the upstream CORE region and the C1 region comprising the CCAAT sequence are important for transcriptional regulation [Resendez, E. et al., *Mol. Cell. Biol.* 8, 4579-4584 (1988); Wooden, S.K. et al., *Mol. Cell. Biol.* 11, 5612-5623 (1991); Li, W.W. et al., *Mol. Cell. Biol.* 14, 5533-5546 (1994)] (see Figure 1); however, its transcriptionally regulatory sequence yet remains undetermined. In the case of yeasts, the

- 4 -

transcriptionally regulatory sequence (UPRE sequence; CAGNGTG) of the GRP78 gene of the budding yeast is clarified [Mori, K. et al., *Genes Cells* 1, 803-817 (1996)]. Although a sequence similar to the UPRE sequence
5 is present upstream of the human GRP78 gene, no activity for directing induced transcription by endoplasmic reticulum stress has been detected in any DNA having a sequence similar to that of the UPRE. As mentioned above, the transcriptionally regulatory region involved in an
10 endoplasmic reticulum stress-response of mammals, especially humans, still remains undetermined.

In cancer cells, endoplasmic reticulum chaperones are expressed at high levels. For example, there has been reported that good correlation exists between
15 intracellular GRP78 level and a tumor size [Cai, J.W. et al., *J. Cell. Physiol.* 154, 229-237 (1993)], and that when GRP78 expression is suppressed by the antisense method, sensitivity to cytotoxic T-cell (CTL) and tumor necrosis factor (TNF) increases [Sugawara, S. et al., *Cancer Res.*
20 53, 6001-6005 (1993)] and take is poor in the mouse, and even if taking, it soon results in regression [Jamora, C. et al., *Proc. Natl. Acad. Sci. USA* 93, 7690-7694 (1996)].

Also, it has been shown that ORP150 is strongly induced in macrophages which infiltrate into
25 arteriosclerotic lesions, and that macrophages treated

- 5 -

with an antisense oligonucleotide to suppress ORP150 expression show decreased viability when exposed to hypoxic conditions, especially in the presence of a denatured LDL (low-density lipoprotein) [Tsukamoto, Y. et al., *J. Clin. Invest.* 98, 1930-1941 (1996)]. Since the macrophages in arteriosclerotic lesions release cytokines, such as tumor necrosis factor, interleukin-1 (IL-1), interleukin-6 (IL-6), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF- β), the macrophages are positioned at the center of intercellular response in arteriosclerotic lesions and assumed to play a major role in the progression of arteriosclerosis.

Cystic fibrosis is a hereditary disease caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the most prevalent of such a mutation being the deletion of phenylalanine at residue 508 (Δ 508F) [Welsh, M.J. and Smith, A.E., *Cell* 73, 1251-1254 (1993)]. CFTR Δ 508F undergoes abnormal sugar chain addition and is degraded without being transported from the endoplasmic reticulum to the Golgi. However, at low temperatures, such Δ 508F mutants leak from endoplasmic reticulum, are localized in the cell membrane, thereby exhibiting activity [Denning, G.M. et al., *Nature* 358, 761-764 (1992)]. Appropriately modifying the stringency

- 6 -

of quality control for nascent membrane proteins in the endoplasmic reticulum would enable the localization and functioning of CTFRΔ508F in the cell membrane.

Furthermore, there have been shown that the mRNA of
5 GRP78, as well as of HSP70, is induced in rat cerebral
ischemia [Wang, S. et al., *Neurochem. Int.* 23, 575-582
(1993); Higashi, T. et al., *Brain Res.* 650, 239-248
(1994)], that the mRNA of GRP78 and GRP94 is induced in
the hippocampal dentate gyrus when convulsive seizures are
10 induced with kainic acid [Lowenstein, D.H. et al., *Mol.*
Brain Res. 22, 299-308 (1994); Little, E. et al.,
Neuroscience 75, 209-219 (1996)], and that ORP150 is
induced in the ischemic mouse brain [Kuwabara, K. et al.,
J. Biol. Chem. 271, 5025-5032 (1996)]. Therefore, the
15 endoplasmic reticulum chaperones are assumed to
protectively act on damaged neurocytes by cerebral
ischemia or the like.

In addition, in wounded tissues and ulcerative
tissues, endoplasmic reticulum chaperones, like HSP, are
20 expected to play an important role in repairing the
damaged tissues.

On the other hand, when a foreign gene is introduced
into a desired cell, to produce a useful protein, the
expression of the introduced gene must be controlled, if
25 the desired gene product exhibits cytotoxicity or affects

- 7 -

cell function. In addition, when a foreign, useful protein is expressed in a host using a recombinant DNA, the desired protein in many cases fails to retain the correct conformation so that the protein cannot be expressed at high levels. It is suggested that the amount of the endoplasmic reticulum chaperones and folding enzymes in usual hosts may be insufficient to ensure ample protein expression and formation of correct conformation.

Therefore, there is a demand for a technique for enabling efficient control of the expression of the endoplasmic reticulum chaperones.

DISCLOSURE OF INVENTION

An object of the present invention is to provide a method for regulating expression of endoplasmic reticulum chaperone genes, which is capable of increasing or decreasing expression of the above gene; a method for expressing a foreign protein; an endoplasmic reticulum stress transcription factor capable of regulating expression of the above gene; and a nucleic acid encoding it, or a complementary nucleic acid thereof.

The above object and other objects of the present invention will be apparent from the following description.

Concretely, the present invention is concerned with the following:

- 8 -

- [1] an endoplasmic reticulum stress transcription factor capable of regulating transcription-inducing activity, wherein the transcription-inducing activity is exhibited by an element having the nucleotide sequence as shown in
5 SEQ ID NO: 1 or an element having a nucleotide sequence having substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1;
- [2] a method for controlling expression of an endoplasmic reticulum chaperone, comprising expressing the endoplasmic
10 reticulum stress transcription factor as defined above in item [1];
- [3] a method for expressing a foreign protein, comprising positively regulating expression of an endoplasmic reticulum chaperone gene by the method as defined in item
15 [2];
- [4] a nucleic acid encoding an activated form of ATF6, or a complementary strand thereto;
- [5] a nucleic acid encoding an activated form of CREB-RP, or a complementary strand thereto;
- 20 [6] a nucleic acid encoding a suppressive form of ATF6, or a complementary strand thereto; and
- [7] a nucleic acid encoding a suppressive form of CREB-RP, or a complementary strand thereto.

25 BRIEF DESCRIPTION OF THE DRAWINGS

- 9 -

Figure 1 shows a nucleotide sequence of the proximal region of human GRP78 promoter [-140 to -19]. The CCAAT-like motifs designated C1 to C5 and TATA sequence are boxed and underlined, respectively. Locations of the CORE and C1 regions, and SICR and CCAAT(N₉)CCACG motifs are also indicated.

Figure 2 shows a tandem repeat structure of the region [-139 to -42] in the human GRP78 promoter. Three repetitive sequences in the above region are aligned, and the nucleotides conserved among at least two repeat sequences are shaded. The ERSE consensus is shown at the top.

Figure 3 shows an ERSE-like sequence (SEQ ID NOs: 5 to 30) of known mammalian and chicken GRP promoters. The nucleotides congruent with the consensus are shaded. In the figure, CRT indicates calreticulin.

Figure 4 shows locations and directions of ERSE-like sequences in various GRP promoters as indicated by closed arrows. CCAAT and TATA sequences are indicated by open arrows and small open boxes, respectively.

Figure 5 shows ERSE-like motifs in vertebrates, plants and fungi. In the figure, CRT indicates calreticulin; PDI, protein disulfide isomerase; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; So, *Spinacia oleracea*; Rc, *Ricinus*

- 10 -

communis; and An, *Aspergillus nigar*.

Figure 6 shows effects of disrupting ERSEs. Panel (A) shows effects of disrupting ERSEs on human GRP78 promoter activities; panel (B) shows effects of disrupting ERSEs on GRP94 promoter activities; and panel (C) shows effects of disrupting ERSEs on calreticulin promoter activities. Each of ERSE1, ERSE2 and ERSE3 of GRP78 promoter, ERSE1 and ERSE3 of GRP94 promoter, and ERSE2 and ERSE3 of calreticulin promoter was disrupted by mutating the respective sequences to gatcT(N₉)aacat, Ctcga(N₉)aacac, gagcT(N₉)aacgc, atgtt(N₉)Agctc, gatcT(N₉)aactc and atgtt(N₉)Agatc, respectively. The intact or resultant mutant promoters were inserted into the KpnI-XhoI sites of the pGL3-Basic vector. Left panel shows a schematic view of intact or mutant ERSE-disruption constructs. Numbers on arrows indicate a nucleotide position from the transcription initiation site. Closed and hatched boxes indicate the locations of an intact ERSE motif and an ERSE motif with remarkable homology to the consensus, respectively. Crosses indicate disrupted ERSEs. Middle panel shows relative firefly luciferase activities by transection into cells. Closed bars and open bars, respectively, indicate HeLa cells transiently transfected with each construct treated with or without 2 µg/ml tunicamycin (in the figure, referred to as "TM") for

- 11 -

16 hours. Right panel shows fold induction of induced activities to basal levels of a reporter activity. The relative luciferase activity and the fold induction were determined from four independent, transient expression assays.

Figure 7 shows involvement of ERSE motifs in transcriptional induction on GRP78, GRP94 and calreticulin. Oligonucleotides encoding each ERSE with indicated flanking sequences as shown in the figure were inserted into the *Bgl*III site of the pGL3-Promoter vector. The relative luciferase activity and the fold induction were determined in the manner as described in Figure 6 by four independent, transient expression assays.

Figure 8 shows nucleotides in ERSE1 from the human GRP78 promoter required for transcriptional induction. Each nucleotide of the segment [-65 to -43] was substituted by transversion (in the figure, substituted nucleotides indicated by lowercase letters). Thereafter, resulting mutated fragment was inserted into the *Xho*I-*Bgl*III sites of the pGL2-Promoter vector. The data are represented as the mean of four independent experiments.

Figure 9 shows nucleotides in ERSE1 from the human GRP78 promoter required for transcriptional induction. Each CCACG and neighboring sequences were changed to those indicated by lowercase letters. Mutated fragments of the

- 12 -

segment [-65 to -38] were inserted into the pGL2-Promoter vector in the same manner as those shown in Figure 8. Insertion mutations each comprising additional A's between CCAAT and CCACG were also shown in lines 38 to 40. The data are represented as the mean of four independent experiments.

Figure 10 shows effects of various ER stress-inducing reagents on ERSE-mediated induction of human GRP78, GRP94 and calreticulin. Cells transiently transfected with the indicated constructs were treated for 16 hours with 2 µg/ml TM (closed bars), 1 µM A23187 (hatched bars) or 100 nM Tg (dotted bars). Each of the constructs as shown in the figure corresponds to the constructs in Figure 6. The data were obtained from four independent experiments.

Figure 11 shows a structure of a reporter plasmid used for one-hybrid screening in yeast.

Figure 12 shows effects of a clone 3-GAL4AD fusion protein on the reporter gene expression. An effector plasmid carrying a gene encoding either GAL4AD protein alone or clone 3-GAL4AD fusion protein was introduced into yeast strain KMY1015 harboring a reporter plasmid of the *lacZ* gene under the control of intact ERSE (5'-CCTTCACCAATCGGCGGCCTCCACGACGG-3') or mutant ERSE (in the figure, mutated nucleotides are indicated in lowercase).

- 13 -

Figure 13 shows comparison of a basic region of human ATF6. Panel (A) shows comparison with yeast Hac1p; Panel (B) shows comparison with members of human ATF/CREB family; and Panel (C) shows comparison with bZIP proteins.

Figure 14 shows effects of ATF6 overexpression on GRP promoters. 100 ng of an effector plasmid carrying a full-length ATF6 cDNA or plasmid vector alone was cotransfected into HeLa cells with 1 µg of a reporter plasmid containing luciferase gene fused with intact or mutant GRP promoters. The mutant promoter constructs used correspond to those of Figure 6. Closed bars and open bars indicated cells treated with or without 2 µg/ml tunicamycin (in the figure, referred to as "TM") for 16 hours, respectively. Experiments were repeated four times.

Figure 15 shows structural homology between ATF6 and CREB-RP. A region showing remarkable homology is indicated by boxes, and the identity is indicated by %.

Figure 16 shows effects of CREB-RP overexpression on GRP promoter. An effector plasmid carrying a full-length CREB-RP gene was cotransfected into HeLa cells harboring a reporter plasmid in the same manner as in Figure 14. Experiments were repeated four times. In the figure, TM indicates tunicamycin.

Figure 17 shows Northern blot hybridization analysis

- 14 -

for ATF6 mRNA. HeLa cells were treated with 2 µg/ml tunicamycin (in the figure, referred to as "TM") for indicated period.

Figure 18 shows immunoblotting analysis of ATF6 protein. *in vitro* translation was carried out using reticulocyte lysate with a control vector (lane 1) or ATF6 cDNA (lane 2). Whole cell extracts were prepared from HeLa cells that had been untreated (lanes 3 and 5) or treated with 2 µg/ml TM for 4 hours (lanes 4 and 6) or from HeLa cells that had been transfected with a control vector (lane 7) or an ATF6 expression plasmid (lane 8). Proteins were detected by using anti-B03N antisera (lanes 1 to 4, 7 and 8) or anti-peptide [anti-ATF6 (N21-34)] antisera (lanes 5 and 6). The positions of the 90 kDa-band (p90ATF6) and 50 kDa-band (p50ATF6) are indicated by open and closed arrows, respectively.

Figure 19 shows the correlation of the appearance of p50ATF6 with the intracellular UPR activity. HeLa cells were treated with 2 µg/ml TM, 7 µM A23187 or 300 nM thapsigargin (in the figure, simply referred to as "Tg") for the indicated period. Alternatively, HeLa cells which were treated separately from the above treated cells were heat-shocked at 43°C for 1 hour and then recovered at 37°C for the indicated period. Whole cell extracts were prepared and analyzed by immunoblotting using an anti-B03N

- 15 -

antiserum or an antiserum specific to either GRP78 or HSP70.

Figure 20 shows immunoblotting analysis for ATF6. HeLa cells cultured in a 60-mm dish so as to have 60%
5 confluency were incubated in the presence of 2 µg/ml tunicamycin (TM) for the indicated period. Cells were washed with PBS, scraped with a rubber policeman, and suspended in 100 µl Laemmli's SDS sample buffer. After boiling the suspension for 5 minutes, aliquots
10 corresponding to 1×10^5 cells were subjected to SDS-PAGE (10% gel) and analyzed by immunoblotting using an anti-ATF6 antibody, or an anti-KDEL antibody recognizing GRP78. The positions of p90ATF6 and p50ATF6 are indicated by open and closed arrows, respectively. The asterisks denote
15 non-specific bands in which a sugar chain is not added to p90ATF6. The positions of molecular weight markers (manufactured by Bio-Rad, prestained SDS-PAGE preparations) are also shown.

Figure 21 shows schematic structures of ATF6 having
20 670 amino acids. The positions of the serine cluster, basic region, and leucine zipper [Zhu et al., *Mol. Cell. Biol.* 17, 4957-4966 (1997)] and the transmembrane domain identified in the present invention are indicated. The bold line represents the region (6th to 307th amino acids)
25 in which ATF6 is fused with *Escherichia coli* maltose-bound

- 16 -

protein in order to prepare an anti-AFT6 antibody. The hydropathy index was calculated by the method of Kyte and Doolittle, *J. Mol. Biol.* 157, 105-132 (1982).

Figure 22 shows the distribution of p90ATF6 and p50ATF6 in each of the fractions of HeLa cells. HeLa cells cultured in a 175-cm² flask so as to have 60 to 80% confluency were incubated in the absence (-) or presence (+) of 2 µg/ml tunicamycin (TM) for 4 hours. Cells were harvested, and disrupted by means of Dounce-type homogenizer, and thereafter the disrupted product was centrifuged at 1,000 x g for 10 minutes to obtain nuclear pellets (in the figure, indicated by N) and supernatant (S) essentially in the same manner as described by Dignam et al. (1983). The resulting supernatant (S) was further centrifuged at 100,000 x g for 1 hour to separate soluble cytosolic fraction (C) from insoluble membrane fraction (M). Aliquots of the indicated fraction as well as unfractionated HeLa cells (whole cell disruption: W) corresponding to 0.5 x 10⁵ cells from which they originated were subjected to SDS-PAGE (10% gel) and analyzed by immunoblotting using anti-ATF6 antibody or various antibodies indicated. The positions of p90ATF6 and p50ATF6 are indicated as open arrows and closed arrows, respectively.

Figure 23 shows a degree of solubilization of

- 17 -

p90ATF6. 1,000 x g supernatant (S) fraction prepared from unstressed HeLa cells obtained in the same manner as in Figure 22 was mixed with 0.1 times by volume of one solution selected from the group consisting of the following solutions: H₂O, 5M NaCl, 1 M Na₂CO₃ (pH 11.0), 10% SDS, 10% Triton X-100, or 10% sodium deoxycholate (DOC). After incubation at room temperature for 15 minutes, the mixture was centrifuged at 100,000 x g for 1 hour to separate supernatant (S) from pellets (P). Thereafter, the resulting sample was subjected to SDS-PAGE (10% gel) and immunoblotted using an anti-ATF6 antibody or an anti-N-terminus of calnexin antibody.

Figure 24 shows topology of p90ATF6. The 1,000 x g supernatant (S) fraction prepared from unstressed HeLa cells (50 µg proteins) was incubated with increasing amounts of trypsin (0 µg for lanes 1, 5 and 9, 0.1 µg for lanes 2, 6 and 10, 0.3 µg for lanes 3, 7 and 11, and 1.0 µg for lanes 4, 8 and 12) at room temperature for 15 minutes. Digestion was terminated by addition of an equal volume of 2 x Laemmli's SDS sample buffer followed by boiling for 5 minutes. Samples were subjected to SDS-PAGE (10% gel), and analyzed by immunoblotting with anti-ATF6 antibody (lanes 1 to 4), anti-N-terminus of calnexin antibody (Calnexin-N, lanes 5 to 8), or anti-C-terminus of calnexin (Calnexin-C, lanes 9 to 12) antibody. The

- 18 -

positions of p90ATF6 are indicated by an arrow. The positions of a full-length calnexin and its truncated form lacking the cytoplasmic domain are shown schematically.

Figure 25 shows schematic structures of full-length ATF6 cDNA, ATF6(670), which is inserted into mammalian expression vector pCGN and various deletion mutants. In the figure, the positions of the basic region, the leucine zipper region and the transmembrane domain are indicated. HA epitope is bound to N-terminal each of various deletion mutants.

Figure 26 shows comparison of mobility on SDS-PAGE for each of various C-terminal deletion mutants with that of p90ATF6 or p50ATF6. HeLa cells in a 60-mm dish were transiently transfected with pCGN vector alone (Vec), expression plasmids for C-terminal deletion mutants pCGN-ATF6(670), pCGN-ATF6(402), pCGN-ATF6(373), pCGN-ATF6(366), or pCGN-ATF6(330). Whole proteins were directly extracted with 1 × Laemmli's SDS sample buffer, and the extract was subjected to SDS-PAGE (10% gel) and analyzed by immunoblotting with an anti-ATF6 antibody. The positions of p90ATF6 and p50ATF6 are indicated by open and closed arrows, respectively.

Figure 27 shows the analytic results of transcription-activating domain of ATF6. Left panel shows schematic structures of ATF6 and fusion proteins between

- 19 -

various ATF6 subregions and the DNA-binding domain (1st to 147th amino acids) of yeast *Gal4p* (GAL4DB). In the figure, the dotted lines denote the region deleted from the construct. The positions of the basic leucine zipper region (bZIP) and the transmembrane domain (TMD) are indicated. Right panel shows transcription activities of various fusion proteins. HeLa cells in a 24-well plate were transiently transfected with each of the fusion protein expression plasmids together with the reporter plasmid pG5luc containing five *Gal4p* binding sites upstream of the firefly luciferase gene. Constitutively expressed luciferase activities were determined and normalized as described in Examples. The relative activities are indicated as the mean \pm standard deviations (bars) from four independent experiments (triplicate determinations). The positive control supplied by the manufacturer (pBIND-Id plus pACT-MyoD control vectors; manufactured by Promega) showed the relative activity of 4.6 ± 0.3 in this assay.

Figure 28 shows effects of overexpression on full-length ATF6, C-terminal deletion mutants and transcription-activating domain deletion mutant on ERSE-mediated transcription. HeLa cells in a 24-well plate were transiently transfected with a reporter plasmid pGL-G78(-132) capable of expressing firefly luciferase

- 20 -

gene under the control of human GRP78 promoter, together with pCGN vector alone (Vec) or each of ATF6 expression plasmids. Transfected cells were treated with (closed bars) or without (open bars) 2 µg/ml tunicamycin (TM) for
5 16 hours prior to measuring luciferase activity. The relative activities are indicated as the mean ± standard deviations (bars) from four independent experiments (triplicate determinations).

Figure 29 shows the results of immunoblot analysis
10 for CREB-RP. In the same manner as the method described in Figure 20, the analysis was carried out by applying a cell extract to SDS-PAGE, and then immunoblotting the resulting gel using an anti-CREB-RP antibody or an anti-ATF6 antibody or an anti-KDEL antibody.

Figure 30 shows effects of overexpression of
15 p60CREB-RP and p50ATF6 on ERSE-mediated transcription. In a 96-well plate, the HeLa cells were transiently transformed with a reporter plasmid pGL-G78(-132) for expressing firefly luciferase gene under the control of a
20 human GRP78 promoter together with pCDNA3.1 vector which has no inserts or with ATF6(1-373) expression plasmid or with CREB-RP(1-389) expression plasmid. Relative activity is indicated by the mean ± standard deviation (bars), based on four independent experiments (each triplicate
25 determinations).

- 21 -

Figure 31 shows effects of overexpression of a deletion mutant of CREB-RP transcription-activating domain on ERSE-mediated transcription. In a 96-well plate, the HeLa cells were transiently transformed using a reporter plasmid pGL-G78(-132) for expressing firefly luciferase gene under the control of a human GRP78 promoter together with pcDNA3.1 vector which has no inserts or with CREB-RP(308-386) expression plasmid. Transfected cells were treated with (closed bars) or without 2 µg/ml tunicamycin (open bars) for 16 hours before luciferase activities were determined. Relative activity is indicated by the mean ± standard deviation (bars), based on four independent experiments (each triplicate determinations).

Figure 32 shows effects of overexpression of deletion mutants of CREB-RP transcription-acting domain, CREB-RP(151-389) and CREP-RP(81-389) on ERSE-mediated transcription. In the figure, closed bars and open bars indicate the same as those in Figure 31.

BEST MODE FOR CARRYING OUT THE INVENTION

In the present specification, the term "endoplasmic reticulum chaperone" refers to a series of proteins characteristic of endoplasmic reticulum, wherein expression of the above proteins is induced by endoplasmic

- 22 -

reticulum stress, and the above proteins are a protein or enzyme having an activity for promoting or catalyzing folding or formation of conformation of secretory proteins and membrane proteins. The endoplasmic reticulum chaperone includes, for example, GRP78, GRP94, ORP150, ERp72, GRP58, calreticulin, PDI, FKBP13, and the like. The endoplasmic reticulum chaperone also encompasses proteins derived from animals such as mammals, and proteins derived from plants.

10 In addition, a gene encoding the above endoplasmic reticulum chaperone refers to herein as "endoplasmic reticulum chaperone gene."

(1) Endoplasmic Reticulum Stress Transcription Factor
15 Capable of Regulating Expression of Endoplasmic Reticulum Chaperone Gene and Nucleic Acid Encoding It
The phrase "endoplasmic reticulum stress transcription factor capable of regulating expression of endoplasmic reticulum chaperone genes" refers to a factor capable of regulating expression by interaction with an endoplasmic reticulum stress-response element (hereinafter simply referred to as "ERSE") which is present on the above endoplasmic reticulum chaperone gene. Therefore, the endoplasmic reticulum stress transcription factor of
20 the present invention can exhibit an excellent effect for
25

- 23 -

regulating expression of the above endoplasmic reticulum chaperone genes at once.

Here, the term "ERSE" refers to an element having activity for directing induced transcription by
5 endoplasmic reticulum stress, wherein the element is an element having a nucleotide sequence as shown in SEQ ID NO: 1 or an element having a nucleotide sequence having substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1.

10 Concretely, the above endoplasmic reticulum stress transcription factor includes an endoplasmic reticulum stress transcription factor capable of regulating transcription-inducing activity, wherein the transcription-inducing activity is exhibited by an element
15 having the nucleotide sequence as shown in SEQ ID NO: 1, or an element having a nucleotide sequence having substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1.

The phrase "nucleotide sequence having substitution
20 of 1 to 3 bases with other kind of bases" means a naturally occurring nucleotide sequence having substitution of 1 to 3 bases with other kind of bases and a nucleotide sequence resulting from artificial substitution of 1 to 3 bases with other kind of bases.

25 The endoplasmic reticulum stress transcription factor

- 24 -

includes, for example, bZIP transcription factor,
concretely ATF6 [Hai, T.W. et al., *Genes Dev.* 3, 2083-2090
(1989)], CREB-RP [Min, J. et al., *Genomics* 30, 149-156
(1995)], XBP-1/TREB5 [Liou, H.C. et al., *Science* 247,
5 1581-1584 (1990), Yoshimura, T. et al., *EMBO J.* 9,
2537-2542 (1990)], and the like.

The nucleotide sequence and the amino acid sequence
of the above ATF6 are shown in SEQ ID NOs: 31 and 32,
respectively. In addition, the nucleotide sequence and
10 the amino acid sequence of the above CREB-RP are shown in
SEQ ID NOs: 33 and 34, respectively.

The endoplasmic reticulum stress transcription factor
encompasses a factor capable of regulating transcription-
inducing activity which is exhibited by the above
15 endoplasmic reticulum stress-response element, wherein the
factor comprises a polypeptide which can be encoded by a
nucleic acid selected from the group consisting of:

- (A) a nucleic acid having the nucleotide sequence as
shown in SEQ ID NO: 31;
- 20 (B) a nucleic acid having the nucleotide sequence as
shown in SEQ ID NO: 33;
- (C) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of one or
more bases in the nucleotide sequence of the nucleic acid
25 (A) or (B); and

- 25 -

(D) a nucleic acid capable of hybridizing under stringent conditions to the strand complementary to any one of nucleic acids (A) to (C).

In the present specification, the phrase "having substitution, deletion, addition or insertion of one or more bases" refers to a state in which one or more bases are naturally or artificially substituted, deleted, added or inserted. In addition, "one or more bases" can be selected in a range capable of regulating transcription-inducing activity exhibited by an endoplasmic reticulum stress-response element.

Further, the term "nucleic acid" refers, for example, to a DNA, and an RNA corresponding thereto.

The term "stringent conditions" includes, for example, conditions described in *Molecular Cloning: A Laboratory Manual, Second Edition* [Sambrook, J. et al. (1989)], and the like.

The endoplasmic reticulum stress transcription factor further encompasses, as long as the factor is capable of regulating transcription-inducing activity exhibited by an endoplasmic reticulum stress-response element, a polypeptide comprising an amino acid sequence having substitution, deletion, addition, or insertion of one or more amino acids in the amino acid sequence as shown in each of SEQ ID NOs: 32 and 34.

- 26 -

In the present specification, "having substitution, deletion, addition, or insertion of one or more amino acids" refers to a state in which one or more amino acids are naturally or artificially substituted, deleted, added
5 or inserted. In addition, "one or more amino acids" can be selected in a range capable of regulating transcription-inducing activity exhibited by an endoplasmic reticulum stress-response element.

In addition, the present invention encompasses an
10 endoplasmic reticulum stress transcription factor which can be obtained as follows.

A method for obtaining an endoplasmic reticulum stress transcription factor capable of enhancing expression of endoplasmic reticulum chaperone genes
15 (hereinafter referred to as "activated form of an endoplasmic reticulum stress transcription factor") includes, but not particularly limited to, a method comprising comparing an expression level of a reporter gene which is incorporated downstream of ERSE described as
20 follows in the absence or presence of endoplasmic reticulum stress by a one-hybrid method using ERSE and a yeast host, whereby obtaining the factor using an increase of the expression level as an index.

On the other hand, an endoplasmic reticulum stress
25 transcription factor for decreasing expression of

- 27 -

endoplasmic reticulum chaperone genes (hereinafter referred to as "suppressive form of an endoplasmic reticulum stress transcription factor") can be obtained by comparing an expression level of a reporter gene which is
5 incorporated downstream of ERSE in the absence or presence of endoplasmic reticulum stress, whereby obtaining the factor using a decrease in the expression level as an index in the same manner as the above.

The activated form of an endoplasmic reticulum stress
10 transcription factor includes, for example, ATF6, and the like.

It is suggested in the following examples of the present specification that the above ATF6 is a membrane protein of the endoplasmic reticulum, and the ATF6 is a
15 bZIP transcription factor having a characteristic of increasing expression of an endoplasmic reticulum chaperone. Transcription-increasing activity of endoplasmic reticulum chaperone genes in which ERSE having intact CCAAT and CCACG region of the nucleotide sequence
20 as shown in SEQ ID NO: 1 is present can be demonstrated by the presence of the above ATF6.

Although the ATF6 is usually a protein having a molecular weight of about 90 kDa (hereinafter referred to as "p90ATF6"), the p90ATF6 is converted into a 50 kDa
25 activated form of protein (hereinafter referred to as

- 28 -

p50ATF6) in the presence of endoplasmic reticulum stress. It is strongly suggested in the following examples of the present specification that the above p50ATF6 is a portion for N-terminal region of p90ATF6, and is localized in the
5 nucleus. p50ATF6 is preferable because the p50ATF6 enhances an activity for increasing expression of endoplasmic reticulum chaperone genes.

The term "endoplasmic reticulum stress" refers to phenomena of accumulation of unfolded proteins in the
10 endoplasmic reticulum under various conditions preventing correct folding of proteins. Concretely, the endoplasmic reticulum stress can be caused by a treatment with, for instance, glucose depletion, tunicamycin [Kozutsumi, Y. et al., *Nature* 332, 462-464 (1988)], calcium ionophore A23187
15 [Watowich, S.K., *Mol. Cell. Biol.* 11, 5612-5623 (1991)], thapsigargin [Li, W.W., *J. Biol. Chem.* 268, 12003-12009 (1993)], 2-deoxyglucose, hypoxia and the like.

Conditions for obtaining p50ATF6 by the above endoplasmic reticulum stress are not particularly limited.
20 For example, when tunicamycin is used, p90ATF6 can be converted into p50ATF6 by treating cells with 0.5 to 8 µg/ml tunicamycin for 2 to 8 hours to induce endoplasmic reticulum stress.

In addition, the activated form of ATF6 includes a
25 polypeptide comprising an N-terminal region (an entire or partial portion of a region of 1st to 373rd amino acids or a

- 29 -

region of 1st to 366th amino acids). From the viewpoint of sufficiently exhibiting activity for increasing expression of endoplasmic reticulum chaperone genes, the activated form of ATF6 includes a polypeptide comprising preferably a
5 region of 1st to 43rd amino acids, more preferably a region of 1st to 150th amino acids. Such an activated form of ATF6 can be prepared, for instance, by introducing stop codon at the position of termination for a desired amino acid region.

Alternatively, examples of the suppressive form of an
10 endoplasmic reticulum stress transcription factor include suppressive form of ATF6, CREB-RP, and the like. Those factors have a property for decreasing expression of endoplasmic reticulum chaperone genes.

The above suppressive form of ATF6 is a polypeptide
15 resulting from disruption of an entire or partial portion of a region of 1st to 150th amino acids from ATF6 or activated form of ATF6. Such a suppressive form of ATF6 has properties of acting as dominant negative, and decreasing expression of endoplasmic reticulum chaperone genes.

20 Here, the term "disruption of an entire or partial portion" refers to a state in which the function possessed by the activated form of the transcription factor cannot be exhibited by mutations such as deletion, insertion, substitution, and the like.

25 Although the above CREB-RP is usually a 110 kDa protein (hereinafter referred to as "p110CREB-RP"), the

- 30 -

p110CREB-RP is converted into a 60 kDa protein
(hereinafter referred to as "p60CREB-RP") in the presence
of endoplasmic reticulum stress, in the same manner as
conversion of p90ATF6 into p50ATF6 by endoplasmic
5 reticulum stress. Surprisingly, differing from
p110CREB-RP, p60CREB-RP has an activity for increasing
expression of endoplasmic reticulum chaperone genes and
acts as an activated form of an endoplasmic reticulum
stress transcription factor. That is, in addition to the
10 above ATF6 (p90ATF6 and p50ATF6), the activated form of an
endoplasmic reticulum stress transcription factor includes
p60CREB-RP.

Furthermore, a polypeptide resulting from deletion of
an entire or partial portion of a region of 1st to 307th
15 amino acids from the above activated form of CREB-RP has
properties of acting as dominant negative, and is included
in the suppressive form of an endoplasmic reticulum stress
transcription factor.

The presence or absence of expression of endoplasmic
20 reticulum chaperone genes by the above endoplasmic
reticulum stress transcription factor (for example, bZIP
transcription factor, and the like) can be confirmed, for
instance, by quantifying mRNA to evaluate the presence or
absence of transcription. In other words, whether or not
25 the desired gene is transcribed can be determined by
extracting an RNA from cells, and subjecting the resulting

- 31 -

RNA to Northern blot hybridization or RNA protection assay. The transcription level of the corresponding mRNA also can be relatively evaluated by introducing a plasmid resulting from incorporation of a fusion gene of the above
5 endoplasmic reticulum chaperone gene with a reporter gene such as chloramphenicol acetyltransferase (hereinafter referred to as "CAT") gene or luciferase gene; and then measuring an activity for the reporter gene product CAT or luciferase.

10 The above endoplasmic reticulum stress transcription factor (for example, bZIP transcription factor, and the like) can be expressed, according to conventional methods, by using cells resulting from incorporation of a DNA comprising a gene encoding the above endoplasmic reticulum
15 stress transcription factor into a chromosome of a host cell; or cells resulting from incorporation of the same DNA into a vector commonly used, and thereafter introducing the resulting vector into a host cell.

There may be used a gene encoding endoplasmic
20 reticulum stress transcription factor in which suitable promoter, and the like are placed. The above promoter includes, for example, SV40 promoter, cytomegalovirus promoter, retrovirus LTR promoter, β -actin promoter, yeast ADH1 promoter, yeast GAP-DH promoter, and the like.

25 When the above gene is incorporated into a vector,

- 32 -

the above vector commonly used includes plasmids, cosmids, viruses, and the like. Concretely, the vector includes, but not limited to, pKCR, pCDL-SR α , pCAGGS, retrovirus vector, adenovirus vector, adeno-associated virus vector, 5 vectors for yeast: YIp, YCp, YE ϕ , YRp derivative plasmids, and the like.

The above host, but not limited to, includes for example, HeLa cells, CHO cells, FM3A cells, L cells, BALB/c3T3 cells, BHK cells, ES cells, yeast *Saccharomyces* 10 *cervisiae*, and the like.

A method for introducing a DNA carrying a gene encoding the above endoplasmic reticulum stress transcription factor into a host cell includes, but not limited to, conventional methods such as calcium phosphate 15 method, electroporation method, lipofection method, and DEAE dextran method.

In the endoplasmic reticulum transcription factor of the present invention, there may be a case where the extent of exhibiting the effect of increase or decrease in 20 expression is different depending upon the ERSE nucleotide sequence in the gene.

Examples of the ERSE include an element [CCAAT(N)₉CCACG] having the nucleotide sequence as shown in SEQ ID NO: 1. By analyzing the transcriptional regulatory 25 region (SEQ ID NO: 4, Figure 1) of a gene encoding a kind

- 33 -

of GRP, human GRP78 protein, the above sequence was clarified for the first time that the sequence is a region involved in the transcriptional regulation by endoplasmic reticulum stress and obtained thereby.

5 The nucleotide sequence as shown in SEQ ID NO: 1 is a well preserved sequence in ERSE1 (SEQ ID NO: 5) of human GRP78, ERSE1 (SEQ ID NO: 6) of murine GRP78, ERSE1 (SEQ ID NO: 7) of rat GRP78, ERSE1 (SEQ ID NO: 8) of human GRP94, ERSE1 (SEQ ID NO: 9) of chicken GRP94, ERSE3
10 (SEQ ID NO: 10) of human GRP94, ERSE3 (SEQ ID NO: 11) of chicken GRP94, ERSE3 (SEQ ID NO: 12) of human calreticulin, ERSE3 (SEQ ID NO: 13) of murine calreticulin, and the like.

 In addition, the ERSE is a nucleotide sequence having
15 substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1, which may be an element capable of exhibiting transcription-inducing activity by endoplasmic reticulum stress. The above element may be a DNA resulting from substitution of
20 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ ID NO: 1 by genetic engineering techniques, or it may be a naturally occurring DNA having a nucleotide sequence having substitution of 1 to 3 bases. Examples of the naturally occurring DNAs include ERSE2
25 (SEQ ID NO: 14) of human GRP78, ERSE2 (SEQ ID NO: 15) of

- 34 -

murine GRP78, ERSE2 (SEQ ID NO: 16) of rat GRP78, ERSE3
(SEQ ID NO: 17) of human GRP78, ERSE3 (SEQ ID NO: 18) of
murine GRP78, ERSE3 (SEQ ID NO: 19) of rat GRP78, ERSE2
(SEQ ID NO: 20) of human GRP94, ERSE4 (SEQ ID NO: 21) of
5 human GRP94, ERSE2 (SEQ ID NO: 22) of chicken GRP94, ERSE1
(SEQ ID NO: 23) of human calreticulin, ERSE2
(SEQ ID NO: 24) of human calreticulin, ERSE2
(SEQ ID NO: 25) of murine calreticulin, ERSE1
(SEQ ID NO: 26) of murine ERp72, ERSE2 (SEQ ID NO: 27) of
10 murine ERp72, ERSE1 (SEQ ID NO: 28) of human protein
disulfide isomerase, ERSE2 (SEQ ID NO: 29) of human
protein disulfide isomerase, ERSE1 (SEQ ID NO: 30) of
human GRP58, and the like. In the ERSE, similar sequences
are found in vertebrates, plants, fungi, and the like
15 (Figure 5).

In the ERSE, the expression can be further
effectively controlled by having coexistence of three
elements ERSEs 1 to 3 as shown in SEQ ID NOs: 1 to 3.

A method for obtaining the ERSE is not particularly
20 limited thereto, and the ERSE can be obtained as described
in Examples set forth below.

The nucleic acid for encoding the endoplasmic
reticulum stress transcription factor has an excellent
characteristic that the nucleic acid can be used in gene
25 therapies for diseases such as ischemic diseases and

- 35 -

cancers. For instance, there are expected that the ischemic diseases can be treated by positively controlling the expression of the endoplasmic reticulum chaperone gene (increase in expression level), and conversely the cancers
5 can be treated by negatively controlling the expression (decrease in expression level).

When expression of the endoplasmic reticulum chaperone gene is positively controlled, as the nucleic acid encoding the endoplasmic reticulum stress
10 transcription factor, there can be used a nucleic acid encoding an activated form of ATF6 or a nucleic acid encoding an activated form of CREB-RP.

Concrete examples of the nucleic acid encoding an activated form of ATF6 include any one of nucleic acids
15 selected from the group consisting of:

- (a) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 373 in SEQ ID NO: 32;
- (b) a nucleic acid having a nucleotide sequence encoding
20 an amino acid sequence as shown in amino acid numbers: 1 to 366 in SEQ ID NO: 32;
- (c) a nucleic acid having a nucleotide sequence as shown in base numbers: 69 to 1187 in SEQ ID NO: 31;
- (d) a nucleic acid having a nucleotide sequence as shown
25 in base numbers: 69 to 1166 in SEQ ID NO: 31;

- 36 -

(e) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (a) to (d); and

(f) a nucleic acid capable of hybridizing to a strand

5 complementary to the nucleic acid of any one of (a) to (e) under stringent conditions,

or the complementary strand thereto. In addition,

concrete examples of the nucleic acid encoding an activated form of CREB-RP include any one of nucleic acids

10 selected from the group consisting of:

(g) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 389 in SEQ ID NO: 34;

15 (h) a nucleic acid having a nucleotide sequence as shown in base numbers: 47 to 1213 in SEQ ID NO: 33;

(i) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (g) and (h); and

20 (j) a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (g) to (i) under stringent conditions, or the complementary strand thereto.

When expression of the endoplasmic reticulum
25 chaperone gene is negatively regulated (decrease in

- 37 -

expression level), as the nucleic acid encoding the
endoplasmic reticulum stress transcription factor, there
can be used a strand nucleic acid complementary to the
nucleic acid encoding an activated form of ATF6, a nucleic
acid strand complementary to the nucleic acid encoding an
5 activated form of CREB-RP, a nucleic acid encoding a
suppressive form of ATF6, a nucleic acid encoding a
suppressive form of CREB-RP, and the like.

Concretely, as the complementary strand to the
10 nucleic acid encoding an activated form of ATF6, there can
be cited complementary strand to the nucleic acids
selected from the group consisting of (a) to (f) above.
The complementary strand to the nucleic acid encoding an
activated form of CREB-RP includes complementary strand to
15 the nucleic acids selected from the group consisting of
(g) to (j) above. As the nucleic acid encoding a
suppressive form of ATF6, there can be cited nucleic acids
selected from the group consisting of:

(k) a nucleic acid having a nucleotide sequence encoding
20 an amino acid sequence as shown in amino acid numbers: 151
to 670 in SEQ ID NO: 32;

(l) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 151
to 373 in SEQ ID NO: 32;

25 (m) a nucleic acid having a nucleotide sequence encoding

- 38 -

an amino acid sequence as shown in amino acid numbers: 151 to 366 in SEQ ID NO: 32;

(n) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 2078 in SEQ ID NO: 31;

5 (o) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 1187 in SEQ ID NO: 31;

(p) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 1166 in SEQ ID NO: 31;

10 (q) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (k) to (p); and
(r) a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (k) to (q) under stringent conditions,

15 or the complementary strand thereto. The nucleic acid encoding a suppressive form of CREB-RP includes nucleic acids selected from the group consisting of:

(s) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 308
20 to 386 in SEQ ID NO: 34;

(t) a nucleic acid having a nucleotide sequence as shown in base numbers: 968 to 1204 in SEQ ID NO: 33;

(u) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 151
25 to 389 in SEQ ID NO: 34;

- 39 -

(v) a nucleic acid having a nucleotide sequence as shown in base numbers: 497 to 1213 in SEQ ID NO: 33;

(w) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 81

5 to 389 in SEQ ID NO: 34;

(x) a nucleic acid having a nucleotide sequence as shown in base numbers: 287 to 1213 in SEQ ID NO: 33;

(y) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least
10 one base in the nucleic acid of any one of (s) to (x); and

(z) a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (s) to (y) under stringent conditions,
or the complementary strand thereto.

15 The substances having activity for suppressing expression of the endoplasmic reticulum chaperones [for instance, a suppressive form of ATF6, nucleic acids of (k) to (r), complementary strands to nucleic acids of (a) to (f), a suppressive form of CREB-RP, nucleic acids of (s)
20 to (z), complementary strands to nucleic acids of (g) to (j), and the like] are expected to be used as a therapeutic drug or a prophylactic drug for cancers, arteriosclerosis or cystic fibrosis. In addition, the substances having activity for inducing expression of the
25 endoplasmic reticulum chaperones [for instance, an

- 40 -

activated form of ATF6, nucleic acids of (a) to (f), an
activated form of CREB-RP, nucleic acids of (g) to (j),
and the like] are expected to be used as a therapeutic
drug or a prophylactic drug for ischemic diseases, wounds
5 or ulcers.

When the substance having activity for suppressing
expression of the endoplasmic reticulum chaperones or the
substance having activity for inducing expression of the
endoplasmic reticulum chaperones is used as a therapeutic
10 drug or prophylactic drug, its administration form
includes oral administration, inhalant administration,
intravenous injection, subcutaneous injection, and the
like.

From the viewpoint of easiness in the introduction of
15 the nucleic acid or the complementary strand thereto into
cells, the method for administering the nucleic acid or
the complementary strand thereto of the present invention
includes a method of administration of a construct
resulting from incorporation of the nucleic acid or the
20 complementary strand thereto into a virus vector by means
such as oral administration, inhalant administration,
intravenous injection, subcutaneous injection, and the
like; a method for directly intramuscularly administering
a composition comprising an expression plasmid harboring
25 the nucleic acid or the complementary strand thereto (DNA

- 41 -

vaccine method); liposome method; lipofection method; microinjection method; calcium phosphate method; electroporation method, and the like.

5 The nucleic acid or the complementary strand thereto of the present invention may be chemically modified nucleic acids, including, for instance, phosphothioates, phosphodithioates, trialkylphosphoric acid esters, alkyl phosphonates, alkylphosphoamidates, and the like.

10 (2) Method for Controlling Expression of Endoplasmic Reticulum Chaperone

One of the great features of a method for controlling expression of an endoplasmic reticulum chaperone resides in that the endoplasmic reticulum stress transcription factor, for instance, bZIP transcription factor, or the like) is expressed. According to the method for controlling expression of endoplasmic reticulum chaperones of the present invention, there is exhibited an excellent effect in that treatment or prophylaxis of cancers, arteriosclerosis, cystic fibrosis, ischemic diseases, wounds or ulcers is made possible by controlling expression of endoplasmic reticulum chaperone genes using the endoplasmic reticulum stress transcription factor. In addition, according to the method for controlling expression of endoplasmic reticulum chaperone genes of the

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- 42 -

present invention, there is exhibited an excellent effect in that a correct conformation can be maintained and a desired protein can be exhibited at a high level in the expression of a foreign protein by a recombinant DNA.

5 Since the endoplasmic reticulum stress transcription factor of the present invention can control expression of all of the endoplasmic reticulum chaperone genes at once, the method using the endoplasmic reticulum stress transcription factor of the present invention is expected
10 to have remarkably excellent controlling effects as compared to the method of controlling expression of each of the individual endoplasmic reticulum chaperone genes.

 In the present invention, expression of endoplasmic reticulum chaperone genes can be controlled by regulating
15 an expression level of the endoplasmic reticulum stress transcription factor within the cell, or the activity for positively or negatively regulating expression of endoplasmic chaperone genes can be adjusted by selecting the endoplasmic reticulum stress transcription factor to
20 be expressed in the cells.

 When expression of the endoplasmic reticulum chaperone genes is controlled by regulating the expression level of the endoplasmic reticulum stress transcription factor, the expression level can be regulated by
25 introducing into cells a DNA encoding the endoplasmic

- 43 -

reticulum stress transcription factor, or a DNA encoding antisense RNA of the endoplasmic reticulum stress transcription factor, or an antisense oligonucleotide.

When expression of the endoplasmic reticulum
5 chaperone genes is regulated positively (increase in expression level) or negatively (decrease in expression level) in accordance with the selection of the endoplasmic reticulum stress transcription factor to be expressed, the expression can be regulated by, for instance, selecting an
10 endoplasmic reticulum stress transcription factor from a bZIP transcription factor ATF6, CREB-RP, XBP-1/TREB5, or the like in accordance with the direction (positive or negative) of the regulation of expression. Besides the above, there may be appropriately selected the endoplasmic
15 reticulum stress transcription factors described in item (1) above (for instance, bZIP transcription factors), or other transcription factors depending upon the characteristics owned by nucleic acids.

When the expression level of the endoplasmic
20 reticulum chaperone gene is increased, for instance, one obtained by expressing AFT6 within the cell as p90ATF6, to form p50ATF6 by endoplasmic reticulum stress may be used, or one obtained by expressing a DNA encoding p50ATF6 may be used. Further, there may be used one obtained by
25 expressing a DNA encoding p60CREB-RP.

- 44 -

In addition, when the ATF6 is used, a polypeptide containing an N-terminal region (an entire or partial portion of a region of 1st to 373th amino acids, or of an entire or partial portion of a region of 1st to 366th amino acids) can be used. From the viewpoint of sufficiently exhibiting activity of increasing expression of the endoplasmic reticulum chaperone genes, there may be employed one obtained by expressing within the cells a DNA encoding a polypeptide preferably containing a region of 1st to 43rd amino acids, more preferably a region of 1st to 150th amino acids.

Further, when the activated form of CREP-RP is used, there may be employed a polypeptide containing an N-terminal region (an entire or partial portion of a region of 1st to 389th amino acids), or one obtained by expressing a DNA encoding the above polypeptide within the cell.

On the other hand, when the expression level of the endoplasmic reticulum chaperone genes is decreased, a polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 150th amino acids from ATF6 or the activated form of ATF6 is used as a dominant negative form, and whereby the expression level of the endoplasmic reticulum chaperone genes can be decreased. In this case, there may be used one obtained

- 45 -

by expressing a DNA encoding the above polypeptide within the cell.

In addition, when the expression level of the endoplasmic reticulum chaperone genes is decreased, a polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 307th amino acids from the activated form of CREB-RP is used as a dominant negative form, and whereby the expression level of the endoplasmic reticulum chaperone genes can be decreased.

In this case, there may be used one obtained by expressing a DNA encoding the above polypeptide within the cell.

(3) Method for Expressing Foreign Protein

There is further provided a method for expressing a foreign protein comprising the method of the present invention for controlling expression of endoplasmic reticulum chaperon genes. The method for expressing a foreign protein is also encompassed in the present invention.

When utilized for control of expression of a foreign gene, 1) a gene encoding the activated form of endoplasmic reticulum stress transcription factor may be simultaneously expressed with a foreign gene located downstream of a promoter for the endoplasmic reticulum chaperone gene, or 2) a gene encoding the activated form

- 46 -

of endoplasmic reticulum stress transcription factor may be simultaneously expressed with a DNA in which an appropriate promoter, a foreign gene, or the like is located downstream of the DNA containing an ERSE portion
5 of the endoplasmic reticulum chaperone gene.

1) Embodiment where a gene encoding the activated form of endoplasmic reticulum stress transcription factor is simultaneously expressed with a foreign gene located
10 downstream of a promoter for the endoplasmic reticulum chaperone gene

In this embodiment, a vector carrying a gene encoding the activated form of endoplasmic reticulum stress transcription factor may be coexpressed with a vector
15 carrying a foreign gene located downstream of the endoplasmic reticulum chaperone gene. Alternatively, a foreign gene may be expressed by introducing the vector carrying the foreign gene into a previously prepared host capable of controlling expression of a gene encoding the
20 activated form of an endoplasmic reticulum stress transcription factor by endoplasmic reticulum stress.

The vector includes, but not particularly limited to, the vectors mentioned above.

The host used for expression includes, but not
25 particularly limited to, the hosts mentioned above.

- 47 -

The method of transfection using a vector includes, but not particularly limited to, the conventional methods mentioned above.

- 5 2) Embodiment where a gene encoding the activated form of an endoplasmic reticulum stress transcription factor is simultaneously expressed with a DNA in which an appropriate promoter, a foreign gene, or the like is located downstream of the DNA containing an ERSE portion
10 of the endoplasmic reticulum chaperone gene

The promoter includes not only promoters derived from human GRP78 gene, human GRP94 gene, and human calreticulin gene, but also any foreign promoters. Concrete examples of the foreign promoters include SV40 promoter,
15 cytomegalovirus promoter, retrovirus LTR promoter, β -actin promoter, and the like.

The DNA comprising the ERSE portion of the endoplasmic reticulum chaperone gene can be located at any positions upstream of the transcriptional initiation
20 point, and it is preferably located within 600 bases from the transcriptional initiation point. Further, the orientation of the DNA may be + or -.

The DNA comprising the ERSE portion of the endoplasmic reticulum chaperone gene can be used for an
25 expression vector by incorporating the DNA into generally

- 48 -

employed plasmids, cosmids, viruses, and the like.

The cells used for expression include the cells mentioned above. In addition, the transfection of the expression vector may be carried out by a conventional method of transfection mentioned above.

EXAMPLES

The present invention will be described in further detail by means of the following Examples, without intending to limit the scope or spirit of the present invention thereto.

Example 1: Isolation of ERSE

(1) Cell Culture

HeLa cells (ATCC CCL2) were cultured in Dulbecco's modified Eagle's medium (glucose at 4.5 g/l) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified 5% CO₂/95% air atmosphere.

(2) Construction of Reporter Plasmids for Isolating ERSE

Recombinant DNA techniques were performed according to the standard procedures described in *Molecular Cloning: A Laboratory Manual* Second Edition, Cold Spring Harbour Laboratory, Published in 1989, and the like.

- 49 -

A 311 bp fragment of a human GRP78 promoter ([-304 to +7] region; the nucleotide position of the transcription initiation site is defined as +1) was amplified by PCR method with genomic DNA of HeLa cells as a template using
5 two oligonucleotides as primers for + strand and - strand, wherein the oligonucleotides were prepared on the basis of the sequence of the human GRP78 gene disclosed by Ting et al. (mentioned above). The resulting amplified fragment was inserted into the *KpnI*-*XhoI* sites of the pGL3-Basic
10 vector (manufactured by Promega) carrying the sequence encoding firefly luciferase but lacking a eukaryotic promoter or enhancer elements, and thereby being cloned.

Deletion fragments having various lengths of the promoter region [-304 to +7] were prepared by PCR method.

15 The resulting amplified fragment was inserted into the *KpnI*-*XhoI* sites of pGL2-Basic vector or pGL2-Promoter vector (manufactured by Promega) carrying SV40 minimal promoter upstream of the sequence encoding firefly luciferase to prepare a reporter promoter for a series of
20 the above deletion mutants.

In order to construct point mutants of the [-139 to -62] and [-65 to -26] regions, synthetic oligonucleotides having appropriate base substitutions were synthesized and annealed, and thereafter the resulting product was ligated
25 to the *KpnI*-*XhoI* sites of pGL2-Promoter vector

- 50 -

(manufactured by Promega). The ERSE sequences in the GRP78 promoter were mutated using Exsite Site-Directed Mutagenesis Kit manufactured by Stratagene. The resulting fragments were inserted into the *KpnI-HindIII* sites of pGL3-Basic vector (manufactured by Promega).

A 397-bp fragment of the human GRP94 promoter ([-363 to +34] region) was amplified by PCR method with genomic DNA of HeLa cells as a template using oligonucleotides as primers, wherein the oligonucleotides were prepared on the basis of the sequence of a human GRP94 gene disclosed by Chang et al. [Chang, S.C. et al., *Mol. Cell. Biol.* 9, 2153-2162 (1989)].

A 511-bp fragment of a human calreticulin promoter region ([-459 to +52] region) was amplified by PCR method with genomic DNA of HeLa cells as a template using oligonucleotides as primers, wherein the oligonucleotides were prepared on the basis of the sequence of a human calreticulin gene disclosed by McCauliffe et al. [McCauliffe, D.P. et al., *J. Biol. Chem.* 267, 2557-2562 (1992)].

These two promoters were used in experiment for disrupting the ERSE sequences. Plasmids purified by conventional cesium chloride (CsCl) method were used in transient transfection.

- 51 -

(3) Transfection Experiments for Transient Expression of Reporter Plasmids for Isolating ERSE

Transfection was carried out by the conventional calcium phosphate method described in the above *Molecular Cloning*, and the like. The HeLa cells were plated onto a 24-well dish so as to have approximately 10% confluency on the day before transfection. One microgram of a reporter plasmid for isolating ERSE and 0.1 µg of a reference plasmid [pRL-SV40 vector (manufactured by Promega) carrying SV40 enhancer and promoter immediately upstream of *Renilla luciferase* gene] were mixed at room temperature in 1 x HEPES buffered saline (composition: 50 mM HEPES, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.08) containing 250 mM CaCl₂ to form CaPO₄-DNA complex. The cells were incubated with the resulting CaPO₄-DNA complex for 16 hours at 37°C, washed with phosphate buffered saline three times, and further incubated in a fresh medium.

(4) Determination of Activity

After culturing the transfection cells obtained in item (3) above for 48 hours, the cells were harvested with a rubber policeman. The harvested cells were suspended in 100 µl lysis buffer (100 mM potassium phosphate buffer, 1 mM dithiothreitol, pH 7.8). Cells were disrupted by freeze-thawing the resulting cell suspension three times,

- 52 -

whereby obtaining a cell-free extract.

In order to induce endoplasmic reticulum stress response, cells were treated with 2 µg/ml tunicamycin 16 hours prior to harvesting the cells.

5 The firefly luciferase and *Renilla* luciferase activities were determined with 5 µl of cell lysate using the Dual Luciferase Reporter Assay Systems (manufactured by Promega) according to the instruction of the manufacturer. The above activities were measured using
10 luminometer (manufactured by Labsystems, trade name: Luminoskan) within a linear range. The relative luciferase activity was found by normalizing the firefly luciferase activity to the *Renilla* luciferase activity.

15 (5) Identification of ERSE

 The endoplasmic reticulum stress response promoter of the mammalian GRP gene was remarked to contain numerous CCAAT motifs. As shown in Figure 3, the structural motif CCAAT-9nt-CCACG is found in the promoters for GRP78, GRP94
20 and calreticulin. As shown in Figures 3 and 4, in these CCAAT sequence and flanking sequences, all of the GRP promoters tested except for FKBP13 have been found to contain multi-copy of similar motifs. As shown in Figure
25 GRP78 promoter actually contains this motif.

- 53 -

Interestingly, as shown in Figure 2, the mammalian GRP78 promoter comprises continuous repeated sequences, each of which contains this motif, and it is deduced that the repeated sequences are caused by the replication during the evolution process. From these findings, it has been suggested that the motif is specifically contained in the mammalian unfolded protein response (UPR). Since the motif is structurally different from UPR responding to the yeast UPR, this motif is referred to as an endoplasmic reticulum stress response element (ERSE). In addition, the ERSE-like sequences are found in vertebrates, plants, fungi, and the like as shown in Figure 5.

(6) Involvement in Transcriptional Induction of ERSE
Motifs of GRP78, GRP94 and Calreticulin

In order to test whether or not the ERSE motif is important in the induction of GRP78, GRP94 and calreticulin, each of the promoters shown on lines 1 to 14 of Figure 6 was ligated to a firefly luciferase gene as a reporter gene, and the ligated product was transfected to HeLa cells in the same manner as in item (3) above. UPR was induced by using tunicamycin (hereinafter simply referred to as "TM"), an inhibitor for N-glycosylation of a protein.

As shown in Figure 6, intact GRP78 promoter has

- 54 -

increased the expression of luciferase in the TM-treated cells (Figure 6, line 2, closed bar) by 5-fold over that of the control (line 2, open bar). The extent of expression was comparable to the one observed in the previous studies [Ting, J. et al., *DNA* 7, 275-286 (1988); Wooden, S.K. et al., *Mol. Cell Biol.* 11, 5612-5623 (1991)] (5 to 7 times); and that of the endogenous GRP78 protein level (5 to 8 times) shown in panel (C) of Figure 19.

In the intact GRP94 promoter and the calreticulin promoter, the luciferase expression was increased by the TM treatment by 8- and 4-fold, respectively, as shown in Figure 6, lines 8 and 12. This expression level agrees with the one previously reported by Ramakrishnan, M. et al., *DNA Cell Biol.* 14, 373-384 (1995); and Waser, M. et al., *J. Cell Biol.* 138, 547-557 (1997).

In addition, as shown on line 3 of Figure 6, when the ERSE1 motif was selectively removed from the GRP promoter, the induction of luciferase was drastically reduced. As shown on lines 4 to 6 of Figure 6, the induction of the luciferase decreased by the removal of the ERSE1 motif was completely abolished by further disruption of ERSE2 and/or ERSE3.

As in the case of GRP78, the disruption of ERSE1 of the GRP94 promoter (shown on line 9 of Figure 6) drastically decreased the induction, and as shown on line

- 55 -

10, the induction was completely disrupted by simultaneous removal of ERSE1 and ERSE3.

As shown on line 13 of Figure 6, the removal of ERSE3 alone at a distal position of the calreticulin promoter
5 had substantially no effect, but as shown on line 14, the disruption of both ERSE3 and ERSE2 of the calreticulin promoter completely prevented induction (line 14).

As shown in Figure 7, when several deduced ERSEs each carrying some flanking sequences (each of ERSEs of GRP78,
10 GRP94 and calreticulin) were at positions upstream of the heterologous promoter (SV40 minimal promoter), since these ERSEs had markedly induced luciferase activity, there has been suggested that these ERSEs are actually functioning.

In addition, the effects by the orientation of ERSE1
15 derived from human GRP78 promoter were evaluated. As a result, the same level of activities was exhibited regardless of its orientation. It was suggested from the above results that the ERSE motif is an essential and sufficient cis-acting element for induction of GRP78,
20 GRP94 and calreticulin and perhaps other GRPs.

In the ERSE motif, in order to determine whether or not the essential nucleotide sequence is actually CCAAT(N)₉CCACG, a point mutation (transversion) was introduced into each of nucleotides of ERSE1 derived from
25 human GRP78 promoter used in the above experiment.

- 56 -

As a result, as shown in Figures 8 and 9, while the substitutions of other nucleotides had substantially no effect, some of the substitutions among the nucleotides [-61 to -57] (Figure 8, lines 7 to 11) or [-46 to -43] (Figure 8, lines 22 to 25) almost completely inhibited the induction. It was strongly suggested from the above results that CCAAT (-61 to -57) and CACG (-46 to -43) were essential for the induction.

As shown in Figure 3, since the nucleotide C[-47] was well conserved, it was further analyzed in detail. In this experiment, a [-65 to -38] segment exhibiting a markedly low response as compared to a [-65 to -42] segment (Figure 9, line 27) was used. While there were substantially no effects when C[-47] was changed to A (see line 30; line 21), as shown on lines 31 and 32 of Figure 9, the induction was completely disrupted when C[-47] was changed to G or T. Therefore, it was shown that C[-47] is also essential for induction. By contrast, there were exhibited substantially no effects in the conversion of A[-42] to other nucleotides. Further, since the insertion of 1 to 3 nucleotides shown on lines 38 to 40 completely disrupted the induction of luciferase, there was shown that the distance between CCAAT (-61 to -57) to CCACG (-47 to -43) is important. These results show that the essential sequence of the ERSE1 motif for the human GRP78

- 57 -

promoter is 19 nucleotides stretch [CCAAT(N)₉CCACG].

(7) Response to Other Endoplasmic Reticulum Stress

Inducer

5 In order to induce experimentally the endoplasmic
reticulum stress response of the mammalian GRP, in
addition to tunicamycin used in all experiments, the cells
were treated with various chemicals including calcium
ionophore A23187 depleting the calcium storage of the
10 endoplasmic reticulum; thapsigargin inhibiting Ca²⁺-ATPase
of the endoplasmic reticulum and excretion of calcium ions
from the endoplasmic reticulum, or the like. The
endoplasmic reticulum stress response of the human GRP
promoter to A23187 and thapsigargin were tested. The
15 results are shown in Figure 10, wherein lines 1, 5, 9, 13,
17 and 21 indicate the relative activity when no inducer
is added as a control; lines 2, 6, 10, 14, 18 and 22
indicate the relative activity when tunicamycin is added;
lines 3, 7, 11, 15, 19 and 23 indicate the relative
20 activity when calcium ionophore A23187 is added; and lines
4, 8, 12, 16, 20 and 24 indicate the relative activity
when thapsigargin is added.

 Since the transcriptional induction by each treatment
of GRP78 (Figure 10, lines 1 to 4), GRP94 (Figure 10,
25 lines 9 to 12), and calreticulin (Figure 10, lines 17 to

- 58 -

20) was completely prevented by the disruption of ERSE as shown on lines 5 to 8, 13 to 16, and 21 to 24 of Figure 10, there was exhibited that ERSE is required for the induction of GRP not only by tunicamycin but also by other inducers.

Example 2: One-Hybrid Screening for cDNA Encoding ERSE Binding Protein

The reporter plasmid for one-hybrid screening was constructed essentially according to the method described by Mori, K. et al. [Genes Cells 1, 803-817 (1996)].

Six tandem repeats of ERSE1 sequence from the human GRP78 promoter [5'-CCTTCACCAATCGGCGGCCTCCACGACGG-3' (SEQ ID NO: 35)] were inserted upstream of the yeast *HIS3* gene ligated to the *IRE1* promoter, while six tandem repeats of mutant ERSE repeats [5'-CCTTCAgactaCGGCGGCCTgatgtACGG-3' (SEQ ID NO: 36)] were inserted upstream of *Escherichia coli lacZ* gene ligated to the *IRE1* promoter. The structure of the above reporter plasmid is schematically shown in Figure 11.

The above reporter plasmids were respectively linearized at the *NcoI* site present in *URA3* gene, and one copy thereof was integrated into the *ura3-52* locus of yeast strain KMY1015 (*MAT α leu2-3, 112 ura3-52 his3- Δ 200 trp- Δ 901 lys2-801 ire1 Δ ::TRP1*) [Mori, K. et al., Genes

- 59 -

Cells 1, 803-817 (1996)]. The resulting yeast strain (hereinafter, referred as KMY1015-ERSE) was used as a host for eliminating unexpected activation via UPR. The KMY1015-ERSE was unable to grow in the absence of
5 histidine and expressed low β -galactosidase activity due to low basal activity of the *IRE1* promoter.

A human lymphocyte cDNA library prepared by using a multicopy plasmid vector carrying the activation domain of yeast transcriptional activator Gal4p (GAL4AD) immediately
10 upstream of the cDNA cloning site was kindly provided by Dr. S.J. Elledge of Baylor College of Medicine through Dr. N. Hayashi of Kanazawa University.

The ERSE-bound protein, when fused with an active domain of yeast transcription factor Gal4p (GAL4AD), is
15 expected to activate the transcription of the reporter gene *HIS3* in an ERSE-dependent manner. Therefore, transformants were prepared using yeast as a host by introducing the above human lymphocyte cDNA library provided by Dr. Elledge, and about 4,300,000 transformants
20 were screened. As a result, 8 clones showing strong His⁺ phenotype were obtained. Among the resulting clones, clones expressing β -galactosidase at a high level despite the absence of the functional ERSE in the upstream region were discarded. As a result, there was obtained clone #3,
25 in which the transcription of the reporter gene increases

- 60 -

in an ERSE-dependent manner.

In order to examine the ERSE-dependency of the transcriptional activation by the clone 3, clone 3-GAL4AD fusion protein was expressed together with various reporter plasmids in which intact ERSEs and mutated ERSEs were placed upstream of the *lacZ* gene ligated with the *IRE1* promoter. The results are shown in Figure 12.

As shown in Figure 12, when the intact ERSE was placed upstream of the reporter gene, the β -galactosidase activity level was remarkably increased by the plasmid expressing clone 3-GAL4AD as an effector (line 5) as compared with the β -galactosidase activity level by the control plasmid expressing GAL4AD alone (line 1).

In addition, the increase by the clone 3-GAL4AD, as shown on lines 6 to 8 of Figure 12, was completely disrupted by using mutated ERSE lacking either one or both of CCAAT or CCACG. The results, as shown in Figures 8 and 9, are relevant to the requirement of intact CCAAT and CCACG for transcriptional activation observed in the HeLa cells.

It is unlikely possibility that ectopic expression of a protein derived from clone 3 in yeast cells causes endoplasmic reticulum stress by the translocation of the clone 3-GAL4AD fusion protein into the endoplasmic reticulum lumen, which in turn results in an enhanced

- 61 -

transcription of a reporter gene directly via yeast UPR pathway in the following two aspects. First, a yeast strain lacking a sensor molecule Ire1p for endoplasmic reticulum stress was used as a host. Second, ERSE is not functional in yeast as a UPR-specific cis-acting element.

As a result of the nucleotide sequence analysis by a conventional method, it was elucidated that clone 3 encodes a known transcription factor, ATF6 [Hai, T.W. et al., *Genes Dev.* 3, 2083-2090 (1989)], a member of the ATF/CREB family containing bZIP motif as a DNA-binding domain.

Interestingly, as shown in Figure 13, the basic region of ATF6 shows marked similarity with a basic region of yeast Hac1p. Although the function of ATF6 is not made evident, ATF6 is originally cloned as a partial cDNA weakly bound to a cAMP response element, and is recently re-isolated as a protein bound to a serum response factor.

Example 3: Isolation of Entire cDNA Encoding ATF6 and CREB-RP

A portion of 5'-region of mRNA for ATF6 thought to be lacking in clone #3 was isolated by 5'RACE using HeLa cells RNA. Here, 5'RACE method employed 5'RACE System (manufactured by Life Technologies Inc.). An intact ATF6 cDNA obtained as described above has a length of 2509 bp,

- 62 -

and a protein having 670 amino acids was encoded (The accession number for GeneBank Data Base is AB015856). The deduced amino acid sequence was different from the sequence reported by Zhu et al. [*Mol. Cell. Biol.* 17, 4957-4966 (1997)] by 4 residues which are deduced to reflect an allele polymorphism.

An entire cDNA of CREB-RP having a similar molecular structure and homology in the amino acid sequence is cloned from HeLa cells RNA by PCR on the basis of the published sequence [Min, J. et al., *Genomics* 30, 149-156 (1995); Khanna, A. et al., *Biochem. J.* 319, 81-89 (1996)].

Example 4: Construction of Effector Plasmid

An entire cDNA was inserted into the *HindIII*-*XhoI* sites or *BamHI*-*EcoRI* sites immediately downstream of the CMV promoter of pCDNA3.1(+) vector (manufactured by Invitrogen) to construct an effector plasmid for expressing ATF6 or CREB-RP, respectively.

Example 5: ERSE-Dependency of Transcriptional Activity

(1) Test for ERSE-Dependency ATF6 on Transcriptional Activity

In order to examine whether or not ATF6 is involved in the transcriptional regulation of the GRP gene in mammalian cells, HeLa cells were co-transfected with an effector plasmid carrying a full-length ATF6 cDNA and a

- 63 -

reporter plasmid resulting from ligation of the firefly luciferase gene to downstream of the intact GRP promoter or a mutant GRP promoter, and GRP promoter activity was evaluated from the luciferase activity. The results are
5 shown in Figure 14.

As indicated by the open bars on lines 1 and 2 in Figure 14, the ATF6 expression plasmid increased the luciferase expression from the GRP78 promoter having intact ERSE sequences by 5-fold over the control level of
10 the aforementioned vector in the absence of TM, but the expression was not affected from the mutant GRP78 promoter lacking all of the three ERSE sequences.

In addition, the ATF6 effector plasmid further increased the β -galactosidase expression from the GRP78
15 promoter having intact ERSE sequences in the presence of TM, as indicated by the closed bars on lines 1 and 2 in Figure 14, while the luciferase expression level did not increase from the mutant GRP78 promoter, as indicated by the closed bars on lines 3 and 4.

20 Similar results were obtained with other GRP promoters: GRP94, calreticulin, ERp72 and GRP58. The results shown on lines 5 through 16 definitely suggested that enhanced expression of ATF6 is capable of specifically activating transcription of the GRP genes via
25 ERSE, regardless of the presence or absence of the

- 64 -

endoplasmic reticulum stress.

(2) Test for ERSE-Dependency on CREB-RP Transcriptional Activity

5 As shown in Figure 15, CREB-RP [Min, J. et al.,
Genomics 30, 149-156 (1995)] shows marked similarity to
ATF6 both in the entire structure and in the deduced amino
acid sequence, especially in its basic region, having 21
out of the 23 residues in common, and the remaining 2
10 being similar basic residues. Also, as shown in Figure
13, CREB-RP possesses the highest similarity among the
known members of the human ATF/CREB family.

 Therefore, with these findings in mind, a test was
carried out to determine whether or not CREB-RP
15 overexpression affects the activity of the GRP promoter.
Interestingly, as indicated by the open bars on lines 1
and 2 in Figure 16, co-transfection of the CREB-RP
expression plasmid hardly affected the reporter expression
from the intact GRP78 promoter in the absence of TM, while
20 the expression was markedly suppressed in the presence of
TM.

 In contrast, as shown on lines 3 and 4, CREB-RP
overexpression did not affect the expression from the
mutant GRP78 promoter in the presence or absence of TM.
25 Because similar results were obtained using the GRP94 or

- 65 -

calreticulin promoter, it has been suggested that CREB-RP overexpression prevents the endoplasmic reticulum stress-induced transcription of the GRP gene via an endogenous trans-activator.

5 It is shown from the results of items (1) and (2) in Example 5 that the closely related transcription factors ATF6 and CREB-RP have definitely opposite effects on induction of target genes, and it was also objected to the possibility that ATF6 overexpression simply activated GRP
10 transcription indirectly by producing endoplasmic reticulum stress in HeLa cells.

Example 6: ATF6 Regulation by Endoplasmic Reticulum Stress

A test was carried out to determine whether or not
15 ATF6 expression is regulated by endoplasmic reticulum stress on an mRNA level or protein level.

(1) Northern Blot Hybridization

Northern blotting was carried out in accordance with
20 the standard method described in *Molecular Cloning: A Laboratory Manual, Second Edition* [Sambrook, et al. (1989)]. Concretely, 10 µg of a poly-A⁺ RNA, prepared from HeLa cells using oligo(dT) magnetic particles (manufactured by Dynabeads), was subjected to
25 electrophoresis on 1% agarose gel containing 2.2 M

- 66 -

formaldehyde, transferred to a nylon membrane, and hybridized with a radiolabeled cDNA specific to either ATF6 or GAPDH.

As shown in Figure 17, Northern hybridization analysis revealed the presence of a single band for 2.5 kb ATF6 mRNA in untreated HeLa cells, as reported by Zhu et al. [Zhu, C. et al., *Mol. Cell Biol.* 17, 4957-4966 (1997)]. In addition, unlike the recently identified splicing system of a yeast HAC1 mRNA, which is specifically induced by endoplasmic reticulum stress [Cox, J.S. et al., *Cell* 87, 391-404 (1996); Kawahara, T. et al., *Mol. Biol. Cell* 8, 1845-1862 (1997)], neither an expression level nor a size of ATF6 mRNA was affected by TM treatment.

15

(2) Preparation of Antisera and Immunoblotting

Two types of antisera against ATF6, i.e., anti-B03N and anti-ATF6 (N21-34), were prepared. The anti-B03N antiserum was obtained by immunizing a rabbit with a fusion protein of N-terminal portion (6th to 307th residues) of ATF6 with the *Escherichia coli* maltose-binding protein, which had been expressed in *Escherichia coli* cells and purified. The anti-B03 antiserum thus obtained was treated with CH-Sepharose 4B (manufactured by Amersham Pharmacia Biotech) on which the

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- 67 -

soluble proteins of *Escherichia coli*, previously transformed with the maltose-binding protein expression plasmid pMAL-c2 (manufactured by New England Biolab), were immobilized, to yield a flow-through fraction for the purified ATF6 antibody. The anti-ATF6(N21-34) antiserum was obtained by immunizing with the keyhole limpet hemocyanin conjugate-synthesized peptide of 14 N-terminal amino acids (21st to 34th residues) of ATF6. The anti-GRP78 and anti-HSP70 antisera were obtained from Stressgen Biotechnologies Corporation.

in vitro translation of ATF6 was carried out using ATF6 cDNA and the TNT T7 Quick Coupled Transcription/Translation System (manufactured by Promega).

By lysing 1×10^6 HeLa cells in 60 μ l of 1 x sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 350 mM dithiothreitol and 0.01% bromophenol blue), a whole cell extract was prepared. This lysate was boiled according to the standard protocol (*Molecular Cloning: A Laboratory Manual, Second Edition*), aliquot (2 μ l) of which lysate was subjected to electrophoresis on 10% SDS polyacrylamide gel, transferred onto a Hybond ECL filter (manufactured by Amersham Pharmacia Biotech), and reacted with various antisera. A pre-stained SDS-PAGE standard (manufactured by Bio-Rad) was used as a size marker. Using an ECL

- 68 -

Western blotting detection kit (manufactured by Amersham Pharmacia Biotech), each antigen was detected.

ATF6, previously known to be constitutively expressed in both HeLa cells and COS cells [Zhu, C. et al., *Mol. Cell Biol.* 17, 4957-4966 (1997)], was analyzed by immunoblotting. The results are shown in Figure 18.

When *in vitro* translation was carried out using a reticulocyte lysate, ATF6 was detected as a single band of 90 kDa molecular weight by the anti-B03N antiserum, as shown on lane 2 in Figure 18. It was also detected by the anti-peptide [anti-ATF6(N21-34)] antiserum as a single band of 90 kDa molecular weight. These molecular weights were greater than the calculated molecular weight of 74.57 kDa, as in the report of Zhu et al.

In contrast, as shown on lane 3, the anti-B03N antiserum reacted with several kinds of proteins in the extract from untreated HeLa cells. Of these proteins, the mobility of the band indicated by the open arrow corresponded to the band from *in vitro* translated ATF6. When HeLa cells were treated with TM for 4 hours, the 90 kDa band decreased, whereas a new 50 kDa band indicated by the closed arrow appeared instead, as shown on lane 4. No other cross-reacted bands were affected.

Importantly, this 50 kDa protein was also detected by the anti-peptide (anti-ATF6 (N21-34)) antiserum (lane 6).

- 69 -

Furthermore, both the 90 kDa and 50 kDa proteins were constitutively present in the extract from ATF6 overexpressing cells (lane 8) but not in the extract from control cells (lane 7), and no other protein bands were produced in excess under the same conditions. This finding suggests that these two proteins may be actually encoded by ATF6 cDNA. The 90 kDa and 50 kDa proteins were designated as p90ATF6 and p50ATF6, respectively.

10 Example 7: Correlation Between p50ATF6 and Intracellular UPR Activity

 In order to determine whether or not the appearance of p50ATF6 is associated with the intracellular UPR activity, stress-induced p50ATF6 appearance and GRP78 expression were monitored with the passage of time by immunoblotting. The results are shown in Figure 19.

 As shown in Figure 19, increase in GRP78 was detected 8 hours after the TM treatment, whereas increase in p50ATF6 became detectable within 2 hours, reaching a peak at 4 to 8 hours, then showing a decrease. Similarly, p50ATF6 appeared in advance of GRP78 induction even in cells treated with A23187 or thapsigargin (indicated by Tg in the figure).

 However, in cells subjected to heat shock, p50ATF6 was hardly detected, even though the definite induction of

- 70 -

HSP70 was found.

These results strongly suggest that the conversion of p90ATF6 to p50ATF6 is an important regulation process in mammalian UPR.

5

Example 8: Conversion of p90ATF6 to p50ATF6 by Endoplasmic Reticulum Stress

In order to determine whether or not p90ATF6 is directly converted to p50ATF6 by endoplasmic reticulum stress, quantitative changes in p90ATF6, p50ATF6 and the target protein GRP78 in TM-treated HeLa cells were evaluated with the passage of time by immunoblotting using the purified ATF6 antibody.

As shown in Figure 20, p50ATF6 appeared at 2 hours after the TM treatment and continued to be detected until 4 hours (lanes 3 to 5). On the other hand, p90ATF6 decreased with the passage of time after the TM treatment, showing clear reverse correlation with the increase in p50ATF6 (lanes 1 to 6). In addition, increased GRP78 expression was observed from 8 hours after the TM treatment. These results suggest that ATF6 is synthesized as a precursor protein (p90ATF6) and specifically converted to a mature protein (50ATF6) by endoplasmic reticulum stress.

25 In the experiment with respect to the passage of time

- 71 -

shown in Figure 20, in addition to p50ATF6, a new band with a slightly faster mobility than that of p90ATF6 was observed in TM-treated HeLa cells, whereby suggesting the possibility that p90ATF6 was modified by glycosylation and hence associated with the endoplasmic reticulum. In fact, when unstressed HeLa cells were analyzed by the indirect immunofluorescence method using the anti-B03N antibody, fine reticular stained images were observed around the nucleus. This staining pattern was the same as that observed using an anti-KDEL antibody (10C3; manufactured by Stressgen) which recognized the principal endoplasmic reticulum chaperones of GRP78 and GRP94. Furthermore, as a result of the computer-aided hydropathy analysis, it was found that as shown in Figure 21, there exists a hydrophobic region having 21 amino acids, which is long enough to penetrate the membrane once. These results strongly support the idea that p90ATF6 is a membrane protein in the endoplasmic reticulum.

Next, a homogenate of HeLa cells treated with or without TM for 4 hours was fractionated by centrifugation to evaluate the localization of p90ATF6 and p50ATF6 (Figure 22). By the first low-speed centrifugation, the majority of p90ATF6 was recovered in the soluble fraction (lane 5), whereas p50ATF6 was fractionated selectively in the nuclear fraction (lane 4), similarly to the nuclear

- 72 -

protein lamin B. By the subsequent high-speed centrifugation, whole p90ATF6 was collected in the membrane fraction (lane 9). Although the distribution pattern of p90ATF6 was substantially identical to that of calnexin, an endoplasmic reticulum transmembrane chaperone, it completely differed from that of lamin B or the cytosolic protein HSP70. Lamin B and HSP70 were detected using an anti-lamin B antibody (manufactured by Santa Cruz) and an anti-HSP70 antibody (C92F3A-5; manufactured by Stressgen), respectively.

Example 9: Topology of p90ATF6

A test was carried out in order to determine whether p90ATF6 is a peripheral or integral membrane protein. The soluble fraction obtained by low-speed centrifugation was subjected to various treatments, and subsequently fractionated by high-speed centrifugation. Immunoblotting was carried out using the purified ATF6 antibody. As a result, as shown in Figure 23, in a treatment with 0.5 M NaCl or 0.1 M Na₂CO₃ (pH 11), in which peripheral membrane proteins were extracted, p90ATF6 was not released from the membrane as well as the integral membrane protein calnexin. In contrast, p90ATF6 and calnexin were both released in the soluble fraction by a detergent, such as 1% SDS or 1% sodium deoxycholate (DOC). 1% Triton X-100

- 73 -

may cause aggregation of p90ATF6.

Next, the orientation of p90ATF6 to the membrane was examined by trypsin treatment (Figure 24). Trypsin digestion was monitored using calnexin, a type I membrane protein, as a control, and using an antibody (manufactured by Stressgen) recognizing the calnexin N-terminal region in the endoplasmic reticulum lumen (calnexin-N) or the calnexin C-terminal region in the cytosol (calnexin-C). At the trypsin concentrations reducing the amount of full-length calnexin, the appearances of indigestible calnexin fragment were not observed with the anti-calnexin C-antibody (lanes 11 and 12), while the appearance of a fragment of a size corresponding to the calnexin N-terminal region was observed with the anti-calnexin N antibody (lanes 7 and 8). This result demonstrates that the region of the luminal side was resistant to trypsin digestion as expected.

Under these experimental conditions, p90ATF6 disappeared at the lowest trypsin concentration examined, and no fragment corresponding to 50 kDa molecular weight could be detected by the anti-ATF6 antibody recognizing the N-terminal region of ATF6 (lane 2). This result strongly suggests that p90ATF6 is a type II membrane protein with its N-terminal region oriented toward the cytosol.

- 74 -

Example 10: Intracellular Localization of p50ATF6

In order to determine whether or not the intracellular localization status is altered by converting p90ATF6 to p50ATF6, a test was carried out by an indirect immunofluorescence method using HeLa cells transformed with the ATF6 expression plasmid. Plasmid pCGN-ATF6 [Zhu et al., *Mol. Cell. Biol.* 17, 4957-4966 (1997)] capable of expressing full-length ATF6 was obtained from Dr. Prywes of Columbia University (hereinafter referred to as pCGN-ATF6(670)). This plasmid was prepared by inserting ATF6 cDNA to the XbaI site of the expression vector pCGN for animal cells, and expressed ATF6 with the influenza virus hemagglutinin (HA) epitope bound to the N-terminus thereof under the control of the cytomegalovirus promoter. The plasmids pCGN-ATF6(402), pCGN-ATF6(373), pCGN-ATF6(366) and pCGN-ATF6(330), all of which cause the expression of an N-terminal fragment of ATF6, were constructed by preparing the regions encoding amino acids 1st to 402nd, 1st to 373rd, 1st to 366th and 1st to 330th, respectively, by PCR, and inserting them, together with the stop codon (TAG), in the XbaI site of pCGN.

The structures of the ATF6 and various mutants expressed from these plasmids are schematically shown in Figure 25. ATF(402) lacks the majority of the C-terminal luminal domain but retains the transmembrane domain

- 75 -

(V³³⁴-L³⁹⁸), whereas the three mutants ATF6(373), ATF6(366) and ATF6(330) lack both the luminal domain and the transmembrane domain. ATF6(373) comprises the basic region (R³⁰⁸-R³³⁰) and the leucine zipper (L³³⁴-L³⁶⁹) in their entirety. ATF6(366) comprises the entire basic region and the majority of the leucine zipper region. ATF(330) comprises the basic region but completely lacks the leucine zipper region. All these proteins have the HA epitope bound to the N-terminus thereof.

Next, the HeLa cells were transiently transformed with expression plasmids for these ATF6s and various mutants, respectively, and the resulting transformant was analyzed by the indirect immunofluorescence method using an anti-HA epitope antibody (Y11; manufactured by Santa Cruz). The expression of ATF6(670) and ATF6(402), both of which have the transmembrane domain, was found to be localized in the endoplasmic reticulum, showing the same staining pattern as that of the fluorescent staining using an anti-KDEL antibody.

On the other hand, in cells expressing ATF6(373), ATF6(366) or ATF6(330), which are mutant ATF6 members lacking both the luminal domain and the transmembrane domain, the nucleus was clearly stained with the anti-HA antibody. These results strongly suggested that p90ATF6 is embedded in the endoplasmic reticulum membrane, whereas

- 76 -

p50ATF6 is cleaved on the N-terminal side of the transmembrane domain and localized in the nucleus.

Example 11: Deduction of Cleavage Site in p50ATF6

5 Production

 In order to deduce the cleavage site involved in p50ATF6 production in response to endoplasmic reticulum stress, the HeLa cells transfected with various C-terminal deletion mutants prepared in Example 10 were analyzed by immunoblotting. The results are shown in Figure 26. In the cells transfected with pCGN-ATF6(670), the two protein bands (lane 2) detected at a position corresponding to about 50 kDa molecular weight served as appropriate molecular weight markers for p50ATF6. These proteins are assumed to be p50ATF6 produced by proteolysis constitutively activated by overexpression of HA-labeled ATF6(670) in the transfected cells. The bands of fast mobility are assumed to have resulted from the loss of the HA label. It is found from the mobility comparison of various C-terminal deletion mutants on SDS-PAGE that the size of p50ATF6 approximates that of ATF6(373), suggesting that when cells are subjected to endoplasmic reticulum stress, p90ATF6 is cleaved between bZIP and the transmembrane domain to produce p50ATF6.

25

- 77 -

Example 12: Transcription-Activating Domain of ATF6

Various PCR-amplified regions of ATF6 were inserted into the XbaI site of the plasmid pBIND (manufactured by Promega), which expresses the DNA-binding domain (amino acids 1st to 147th, referred to as "GAL4DB") of the yeast transcription factor Gal4p under the control of the cytomegalovirus promoter, to yield plasmids for expressing fusion proteins of the ATF6 fragments with GAL4DB. These expression plasmids were transiently introduced into HeLa cells together with the reporter plasmid pG51uc (manufactured by Promega) containing five Gal4p binding sites just upstream of the adenovirus major late promoter to determine their ability of activating the transcription of the luciferase gene. As shown in Figure 27, the transcription-activating ability was mapped to an N-terminal 150-amino acid region (lines 6 and 9 to 12), especially demonstrating the great contribution of the 43 N-terminal amino acids (lines 6 to 8). The presence of a transcription-activating domain at the N-terminus ensures the function of p50ATF6 as a transcription factor in the nucleus.

Example 13: Transcriptional Activity of C-terminusDeletion Mutants of ATF6

Using HeLa cells co-transfected with the reporter

- 78 -

plasmid pGL3-G78(-132) [see Example 1 (Figure 6, line 2)]
resulting from ligation of the firefly luciferase gene to
downstream of the human GRP78 promoter ([-132 to +7],
numbered on the basis of the translation initiation point
5 taken as +1) and the expression plasmid for the ATF6
C-terminal deletion mutant of Example 10, the effects of
each of the overexpressed C-terminal deletion mutants of
ATF6 on ERSE-mediated transcriptional activation were
evaluated.

10 As a result, it was found that when the full-length
ATF6, namely ATF(670), is transiently overexpressed using
pCGN-ATF6(670), the transcription of the luciferase gene
from the GRP78 promoter having ERSE, is constitutively
activated in the absence of endoplasmic reticulum stress
15 (Figure 28, line 2), as in the results obtained in Example
5 (Figure 14). The higher relative luciferase activity in
comparison with the level obtained in Example 5 (Figure
14) was attributed to the expression of more ATF6 in the
cells transfected with pCGN-ATF6 than in those transfected
20 with pcDNA-ATF6. Further, the constitutive activation of
transcription of the luciferase gene can be well explained
by the fact that a p50ATF6-like protein was constitutively
produced in the aforementioned cells transfected with
pCGN-ATF6(670) (see Figure 26, lane 2). Similarly, the
25 reporter luciferase activity was also constitutively

- 79 -

enhanced by overexpression of endoplasmic
reticulum-localized ATF6(402) [open bar on line 3 in
Figure 28].

The results as shown by the open bars on lines 4 and 5
5 and the open bars on lines 2 and 3 in Figure 28 demonstrate
that increased luciferase activity by 5-fold or more over
that obtained with the endoplasmic reticulum-localized ATF6
ATF(670) or ATF(402) is obtained by overexpressing the
nucleus-localized ATF6 mutants ATF6(373) and ATF6(366).
10 These results agree with the fact that the nuclear protein
p50ATF6 shows the activated form of ATF6.

On the other hand, another nucleus-localized
ATF6(330) showed markedly lower activity than ATF6(373) or
ATF(366), despite having nearly the same expression level
15 (Figure 26), demonstrating the importance of the leucine
zipper in the transcriptional activity of ATF6 (compare
line 6 with lines 4 and 5 in Figure 28).

Example 14: Transcription-Activating Domain Deletion

20 Mutants of ATF6

Each of pCGN-ATF6(151-670), pCGN-ATF6(151-402),
pCGN-ATF6(151-373), pCGN-ATF6(151-366) and
pCGN-ATF6(151-330), which expressed mutant ATF6 lacking a
region of amino acids 1st to 150th from the N-terminal of
25 ATF6 or the mutants prepared in Example 10, was

- 80 -

constructed by preparing the regions encoding amino acids
151st to 670th, 151st to 402nd, 151st to 373rd, 151st to
366th and 151st to 330th, respectively, by PCR, and
inserting each region, together with the stop codon, in
5 the XbaI site of pCGN (see Figure 25).

Being expressed from these plasmids, each of
ATF6(151-670), ATF6(151-402), ATF6(151-373), ATF6(151-366)
and ATF6(151-330) resulted from the deletion of the
N-terminal 150 amino acids (M^1-L^{150}) from ATF6(670),
10 ATF6(402), ATF6(373), ATF6(366) and ATF6(330),
respectively.

In the same manner as in Example 10, HeLa cells were
transiently transformed with each expression plasmid for
an ATF6 transcription-activating domain deletion mutant,
15 and the intracellular localization of the ATF6
transcription-activating domain deletion mutant expressed
was evaluated by the indirect immunofluorescence method
using an anti-HA epitope antibody (Y11; manufactured by
Santa Cruz). As a result, ATF6(151-670) and ATF6(151-402)
20 were found to be localized in endoplasmic reticulum, as
were the corresponding ATF6(670) and ATF6(402),
respectively. In addition, ATF6(151-373), ATF6(151-366)
and ATF6(151-330) were all localized in the nucleus, as
were ATF6(373), ATF6(366) and ATF6(330).

25 Next, in the same manner as in Example 13, the effect

- 81 -

of the overexpression of each ATF6 transcription-activating domain deletion mutant on ERSE-mediated transcriptional activation was evaluated by co-transfection with the reporter plasmid pGL3-G78(-132).

5 As a result, as shown on lines 7 to 11 in Figure 28, all ATF6 transcription-activating domain deletion mutants strongly suppressed the expression of the reporter gene to a level equal to or less than that when the control pCGN vector (line 1) is used. ATF6(151-373), ATF6(151-366) and
10 ATF6(151-330) were shown to act dominant-negatively on endogenous p50ATF6 in the nucleus. On the other hand, ATF6(151-670) and ATF6(151-402) were assumed to act dominant-negatively on endogenous p50ATF6 by inducing
15 the endoplasmic reticulum stress by their overexpression in the endoplasmic reticulum, followed by migration into the nucleus of the resulting p50ATF6 lacking the transcription-activating domain.

20 Example 15: Conversion of p110CREB-RP to p60CREB-RP by Endoplasmic Reticulum Stress

 In order to elucidate whether or not a full-length CREB-RP (p110CREB-RP) is converted to p60CREB-RP by endoplasmic reticulum stress, quantitative changes in p110CREB-RP and p60CREB-RP in TM-treated HeLa cells were
25 evaluated with the passage of time by immunoblotting using

- 82 -

a purified CREB-RP antibody. The CREB-RP antibody was prepared by immunizing a rabbit with a fusion protein of a region of amino acids 1st to 307th of CREB-RP with GST, expressed in *Escherichia coli*, and used after absorption with GST and bacterial proteins and affinity purification with the GST-CREB-RP fusion protein.

When the conversion of p90ATF6 to p50ATF6 and the amount of the target protein GRP78 were also examined in the same manner as in Example 8, p60CREB-RP was found to appear 2 hours after the TM treatment, and, unlike p50ATF6, to be persistently expressed thereafter, as shown in Figure 29.

Example 16: Activated Form of CREB-RP

From the CREB-RP expression plasmid prepared in Example 4, a DNA fragment encoding a region of amino acids 1st to 389th of CREB-RP was prepared by PCR, and inserted into the *HindIII* site of the pcDNA3.1(+) vector. Being expressed from this plasmid, CREB-RP(1-389) comprises the N-terminus through the basic region and the leucine zipper region of CREB-RP, corresponding to p60CREB-RP as converted from p110CREB-RP by endoplasmic reticulum stress.

The CREB-RP(1-389) expression plasmid and a reporter plasmid resulting from ligation of the luciferase gene to

- 83 -

downstream of the GRP78 promoter or mutant GRP78 promoter were introduced into the HeLa cells to evaluate the effect of CREB-RP(1-389) overexpression on ERSE-mediated transcriptional activation.

5 As shown in Figure 30, the overexpression of CREB-RP(1-389), which is assumed to correspond to p60CREB-RP, constitutively activated the transcription, in the same manner as in p50ATF6. Because this transcriptional activation is not observed when mutant
10 ERSE is used, it is found to be ERSE-dependent.

Example 17: Suppressive Form of CREB-RP

A DNA fragment encoding a region of amino acids 308th to 386th of CREB-RP, in which Met was added to the
15 N-terminus thereof, was prepared from the CREB-RP expression plasmid prepared in Example 4 by PCR, and the DNA fragment was inserted into the *HindIII* site of the pCDNA3.1(+) vector. Being expressed from this plasmid, CREB-RP(308-386) does not contain the transcription-
20 activating domain present in the N-terminal region of CREB-RP but only selectively comprises the basic region and the leucine zipper region.

In the same manner as in Example 16, the effect of CREB-RP(308-386) overexpression on ERSE-mediated
25 transcriptional activation was evaluated. As shown in

- 84 -

Figure 31, the ERSE-dependent transcriptional induction by the TM treatment was strongly suppressed. The results demonstrate that CREB-RP(308-386) acts dominant-negatively on the endogenous endoplasmic reticulum stress transcription factor.

In addition, in the same manner as in Example 16, the effect of overexpression of CREB-RP(81-389) or CREB-RP(151-389) on ERSE-mediated transcriptional activation was evaluated. Here, each of CREB-RP(81-389) and CREB-RP(151-389) lacks transcription-activating domain. As shown in Figure 32, the ERSE-dependent transcriptional induction by the TM treatment was strongly suppressed. The results demonstrate that each of CREB-RP(81-389) and CREB-RP(151-389) dominant-negatively acts on the endogenous endoplasmic reticulum stress transcription factor.

SEQUENCE LISTING FREE-TEXT

In SEQ ID NO: 1, "n" is A or C or G or T. The sequence as shown in SEQ ID NO: 1 is the ERSE1 consensus sequence.

In SEQ ID NO: 2, "n" is A or C or G or T. The sequence as shown in SEQ ID NO: 2 is the ERSE2 consensus sequence.

In SEQ ID NO: 3, "n" is A or C or G or T. The

- 85 -

sequence as shown in SEQ ID NO: 3 is the ERSE3 consensus sequence.

INDUSTRIAL APPLICABILITY

5 The endoplasmic reticulum stress transcription factor is capable of regulating increase or decrease of expression of endoplasmic reticulum chaperone genes. According to the method for controlling expression of endoplasmic reticulum chaperones of the present invention,
10 the increase or decrease of expression of endoplasmic reticulum chaperone genes can be regulated. Further, treatment or prophylaxis of cancers, arteriosclerosis, cystic fibrosis, ischemic diseases, wounds or ulcers is made possible by the method for controlling expression of
15 endoplasmic reticulum chaperones of the present invention. Moreover, by applying the method for controlling expression mentioned above to expression of a foreign useful protein, there are exhibited excellent effects that the foreign useful protein retains correct conformation,
20 and that the foreign protein can be expressed at a high level.

EQUIVALENT

25 Those skilled in the art will recognize, or be able to ascertain using simple routine experimentation, many

- 86 -

equivalents to the specific embodiments of the invention described in the present specification. Such equivalents are intended to be encompassed in the scope of the following claims.

- 87 -

CLAIMS

1. An endoplasmic reticulum stress transcription factor capable of regulating transcription-inducing activity,
5 wherein said transcription-inducing activity is exhibited by an element having the nucleotide sequence as shown in SEQ ID NO: 1 or an element having a nucleotide sequence resulting from substitution of 1 to 3 bases with other kind of bases in the nucleotide sequence as shown in SEQ
10 ID NO: 1.
2. The endoplasmic reticulum stress transcription factor according to claim 1, wherein said endoplasmic reticulum stress transcription factor is bZIP transcription factor.
15
3. The endoplasmic reticulum stress transcription factor according to claim 2, wherein said bZIP transcription factor is ATF6 (SEQ ID NO: 32) or CREB-RP (SEQ ID NO: 34).
- 20 4. The endoplasmic reticulum stress transcription factor according to claim 2, comprising a polypeptide which can be encoded by a nucleic acid selected from the group consisting of:
(A) a nucleic acid having the nucleotide sequence as shown
25 in SEQ ID NO: 31;

- 88 -

(B) a nucleic acid having the nucleotide sequence as shown
in SEQ ID NO: 33;

(C) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of one or
5 more bases in the nucleotide sequence of the nucleic acid
(A) or (B); and

(D) a nucleic acid capable of hybridizing under stringent
condition to the strand complementary to any one of
nucleic acids (A) to (C).

10

5. The endoplasmic reticulum stress transcription factor
according to claim 2, wherein said bZIP transcription
factor is an activated form of ATF6.

15 6. The endoplasmic reticulum stress transcription factor
according to claim 5, wherein said activated form of ATF6
is p50ATF6 resulting from processing by endoplasmic
reticulum stress.

20 7. The endoplasmic reticulum stress transcription factor
according to claim 5, wherein said activated form of ATF6
is a polypeptide obtainable by expression of DNA encoding
p50ATF6.

25 8. The endoplasmic reticulum stress transcription factor

- 89 -

according to claim 5, wherein said activated form of ATF6 is a polypeptide comprising an entire or partial portion of a region of 1st to 373th amino acids or a region of 1st to 366th amino acids in ATF6.

5

9. The endoplasmic reticulum stress transcription factor according to claim 2, wherein said bZIP transcription factor is an activated form of CREB-RP.

10 10. The endoplasmic reticulum stress transcription factor according to claim 9, wherein said activated form of CREB-RP is p60CREB-RP resulting from processing by endoplasmic reticulum stress.

15 11. The endoplasmic reticulum stress transcription factor according to claim 9, wherein said activated form of CREB-RP is a polypeptide obtainable by expression of DNA encoding p60CREB-RP.

20 12. The endoplasmic reticulum stress transcription factor according to claim 9, wherein said activated form of CREB-RP is a polypeptide comprising an entire or partial portion of a region of 1st to 389th amino acids in CREB-RP.

25

- 90 -

13. The endoplasmic reticulum stress transcription factor according to claim 2, wherein said bZIP transcription factor is a suppressive form of ATF6.

5 14. The endoplasmic reticulum stress transcription factor according to claim 13, wherein said suppressive form of ATF6 is a polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 150th amino acids in ATF6.

10

15. The endoplasmic reticulum stress transcription factor according to claim 13, wherein said suppressive form of ATF6 is a polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 150th
15 amino acids in the activated form of ATF6 of any one of claims 5 to 8.

16. The endoplasmic reticulum stress transcription factor according to claim 2, wherein said bZIP transcription
20 factor is a suppressive form of CREB-RP.

17. The endoplasmic reticulum stress transcription factor according to claim 16, wherein said suppressive form of CREB-RP is a polypeptide resulting from disruption of an
25 entire or partial portion of a region of 1st to 307th

- 91 -

amino acids in CREB-RP.

18. The endoplasmic reticulum stress transcription factor according to claim 16, wherein said suppressive form of
5 CREB-RP is a polypeptide resulting from disruption of an entire or partial portion of a region of 1st to 307th amino acids in the activated form of CREB-RP of any one of claims 9 to 12.

10 19. A method for controlling expression of an endoplasmic reticulum chaperone, comprising expressing the endoplasmic reticulum stress transcription factor as defined in any one of claims 1 to 18.

15 20. The method for controlling expression according to claim 19, comprising regulating an expression level of an endoplasmic reticulum stress transcription factor in a cell.

20 21. The method for controlling expression according to claim 19 or 20, comprising positively or negatively regulating expression of an endoplasmic reticulum chaperone gene by selection of an endoplasmic reticulum stress transcription factor to be expressed.

25

- 92 -

22. A method for expressing a foreign protein, comprising positively regulating expression of an endoplasmic reticulum chaperone gene by the method as defined in any one of claims 19 to 21.

5

23. A nucleic acid encoding the activated form of ATF6 as defined in any one of claims 6 to 8, or the complementary strand thereto.

10

24. The nucleic acid according to claim 16, wherein said nucleic acid is selected from the group consisting of:

(a) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 373 in SEQ ID NO: 32;

15

(b) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 1 to 366 in SEQ ID NO: 32;

(c) a nucleic acid having a nucleotide sequence as shown in base numbers: 69 to 1187 in SEQ ID NO: 31;

20

(d) a nucleic acid having a nucleotide sequence as shown in base numbers: 69 to 1166 in SEQ ID NO: 31;

(e) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (a) to (d); and

25

(f) a nucleic acid capable of hybridizing to a strand

- 93 -

complementary to the nucleic acid of any one of (a) to (e)
under stringent condition,
or the complementary strand thereto.

5 25. A nucleic acid encoding an activated form of CREB-RP
as defined in any one of claims 10 to 12, or the
complementary strand thereto.

26. The nucleic acid according to claim 25, wherein the
10 nucleic acid is selected from the group consisting of:
(g) a nucleic acid having a nucleotide sequence encoding
an amino acid sequence as shown in amino acid numbers: 1
to 389 in SEQ ID NO: 34;
(h) a nucleic acid having a nucleotide sequence as shown
15 in base numbers: 47 to 1213 in SEQ ID NO: 33;
(i) a nucleic acid having a nucleotide sequence having
substitution, deletion, addition or insertion of at least
one base in the nucleic acid of any one of (g) to (h); and
(j) a nucleic acid capable of hybridizing to the strand
20 complementary to the nucleic acid of any one of (g) to (i)
under stringent condition,
or the complementary strand thereto.

27. A nucleic acid encoding the suppressive form of ATF6
25 as defined in claims 14 or 15, or the complementary strand

- 94 -

thereto.

28. The nucleic acid according to claim 27, wherein the nucleic acid is selected from the group consisting of:

- 5 (k) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 151 to 670 in SEQ ID NO: 32;
- (l) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 151
10 to 373 in SEQ ID NO: 32;
- (m) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 151 to 366 in SEQ ID NO: 32;
- (n) a nucleic acid having a nucleotide sequence as shown
15 in base numbers: 519 to 2078 in SEQ ID NO: 31;
- (o) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 1187 in SEQ ID NO: 31;
- (p) a nucleic acid having a nucleotide sequence as shown in base numbers: 519 to 1166 in SEQ ID NO: 31;
- 20 (q) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least one base in the nucleic acid of any one of (k) to (p); and
- (r) a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (k) to (q)
25 under stringent condition,

- 95 -

or the complementary strand thereto.

29. A nucleic acid encoding the suppressive form of CREB-RP as defined in claim 17 or 18.

5

30. The nucleic acid according to claim 29, wherein said nucleic acid is selected from the group consisting of:

(s) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 308

10 to 386 in SEQ ID NO: 34;

(t) a nucleic acid having a nucleotide sequence as shown in base numbers: 968 to 1204 in SEQ ID NO: 33;

(u) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 151

15 to 389 in SEQ ID NO: 34;

(v) a nucleic acid having a nucleotide sequence as shown in base numbers: 497 to 1213 in SEQ ID NO: 33;

(w) a nucleic acid having a nucleotide sequence encoding an amino acid sequence as shown in amino acid numbers: 81

20 to 389 in SEQ ID NO: 34;

(x) a nucleic acid having a nucleotide sequence as shown in base numbers: 287 to 1213 in SEQ ID NO: 33;

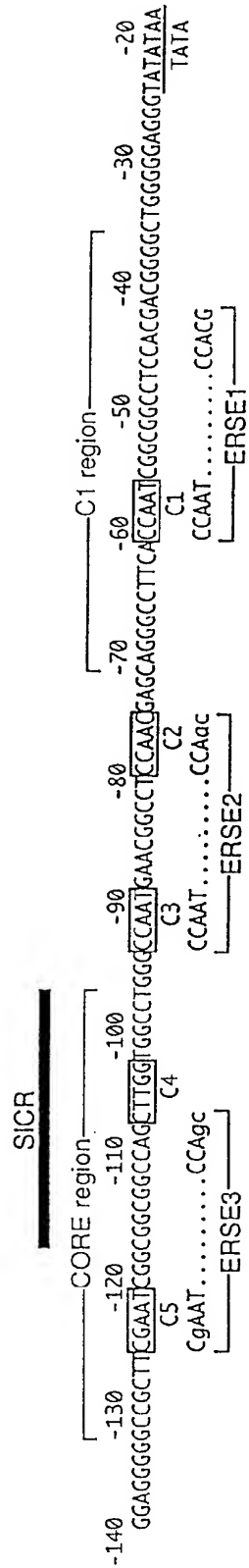
(y) a nucleic acid having a nucleotide sequence having substitution, deletion, addition or insertion of at least

25 one base in the nucleic acid of any one of (s) to (x); and

- 96 -

(z) a nucleic acid capable of hybridizing to the strand complementary to the nucleic acid of any one of (s) to (y) under stringent conditions,
or the complementary strand thereto.

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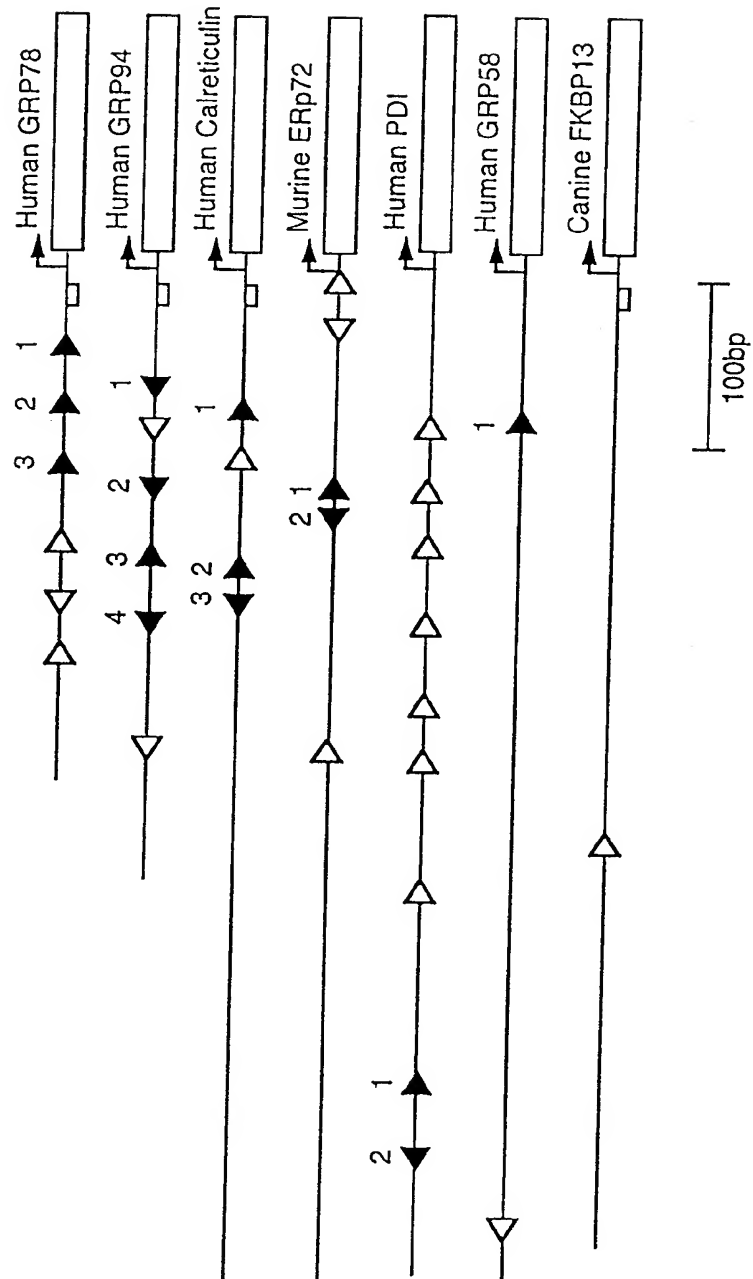
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|-----ERSE-----|
CCAAT.....CCACG

(-139)	GAGGGGGCGCTTGAATCGGCGGCGGCCAGC	(-108)
(-107)	TTGGTGGCGTGGGCGAATGAACGGCGTCCAACG	(-75)
(-74)	AGCAGGGCGTTCACCAATCGGCGGCGTCCACGA	(-42)

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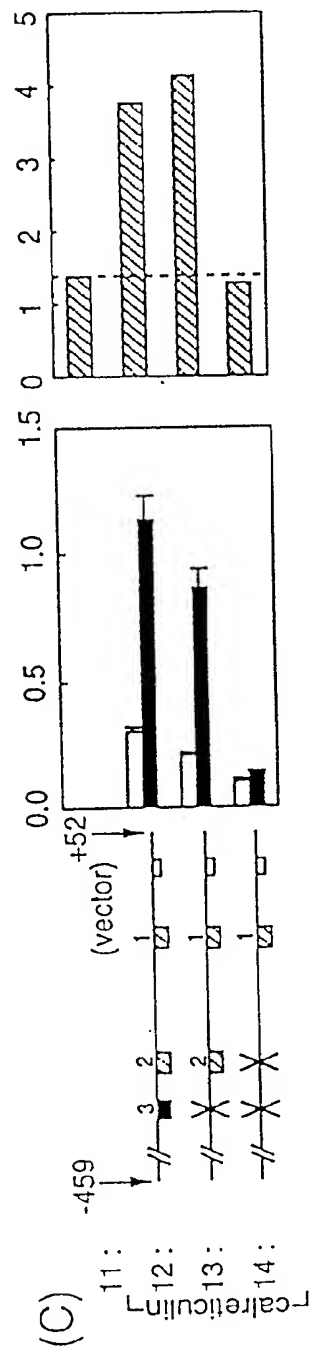
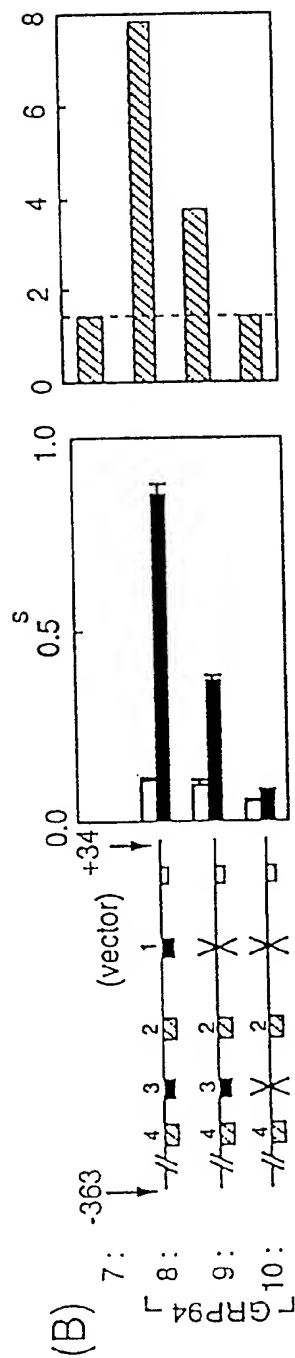
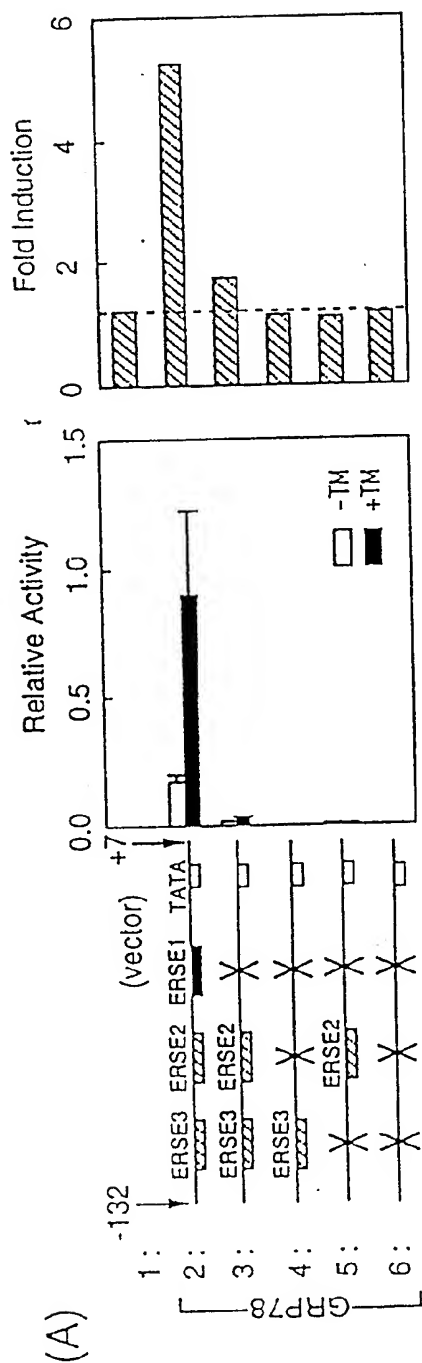
GRP78	ERSE1 (human)	CCAATCGGCGGCCTCCAGG
	ERSE1 (murine)	CCAATCGGAGGCCTCCAGG
	ERSE1 (rat)	CCAATCGGAGGCCTCCAGG
GRP94	ERSE1 (human)	CCAATCGCGCCGCACCAGG
	ERSE1 (chicken)	CCAATGGGAGCGCACCAGG
	ERSE3 (human)	CCAATCGGAAGGAGCCAGG
	ERSE3 (chicken)	CCAATCGACCCGGCCAGG
CRT	ERSE3 (human)	CCAATGATGGTCGACCAGG
	ERSE3 (murine)	CCAATGAGGGTCGACCAGG
GRP78	ERSE2 (human)	CCAATGAACGGCCTCCAAC
	ERSE2 (murine)	CCAATCAGCGGCCTCCAAC
	ERSE2 (rat)	CCAACCAGCGGCCTCCAAC
	ERSE3 (human)	GGAATCGGCGGCGGCCAGC
	ERSE3 (murine)	GGAATCGGCAGCAGCCAGC
	ERSE3 (rat)	GGAATCGGCAGCGGCCAGC
GRP94	ERSE2 (human)	CCAATCGGAGCTGTCCAGG
	ERSE2 (chicken)	CCAATCGTGGCTTTCCATG
	ERSE4 (human)	CCAATCAAATGGCTCCGGG
CRT	ERSE1 (human)	CCAATGACAAAGTGGCAGG
	ERSE2 (human)	CCAATAGAAATCGGCCATC
	ERSE2 (murine)	CCAATAGAAATCAGCCATC
ERp72	ERSE1 (murine)	CCAATCAGAAGGGGGCACC
	ERSE2 (murine)	CCAATCACGGGCTGCCAGT
PDI	ERSE1 (human)	CCAGTCAGAATGCAACACG
	ERSE2 (human)	CCAAC TGGCACGCCCECCG
GRP58	ERSE1 (human)	CCAATCAGCGGCTGCCACA

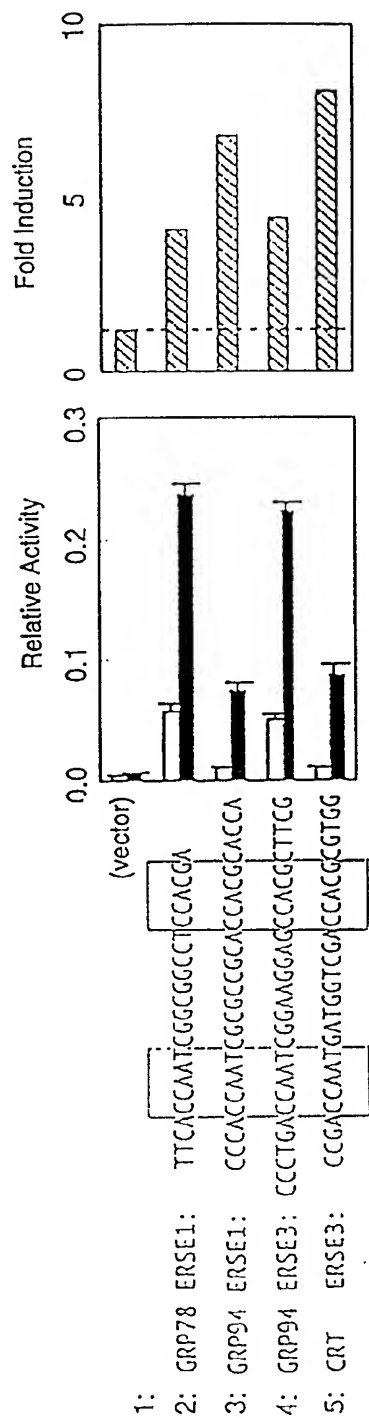


F I G . 4

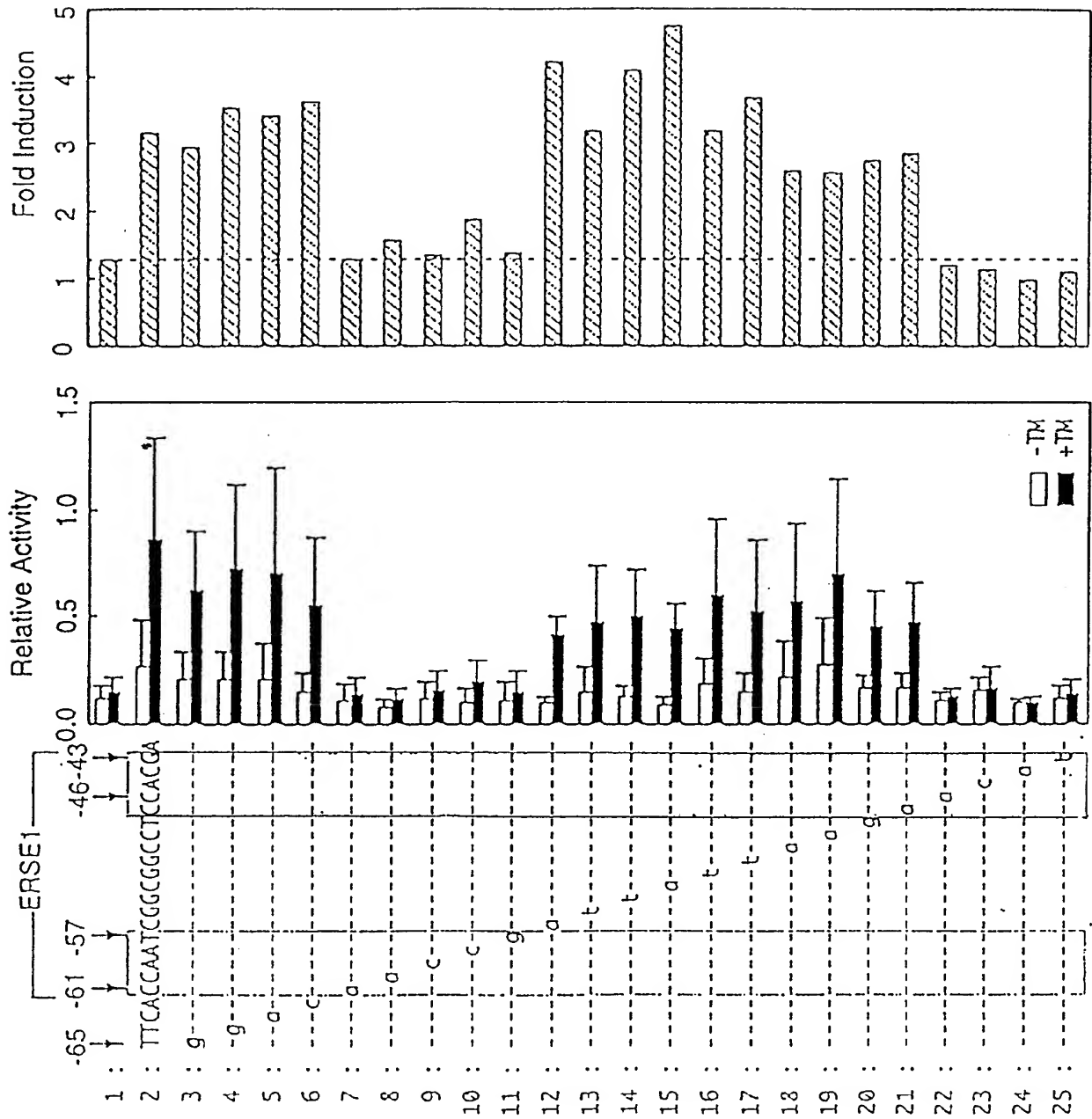
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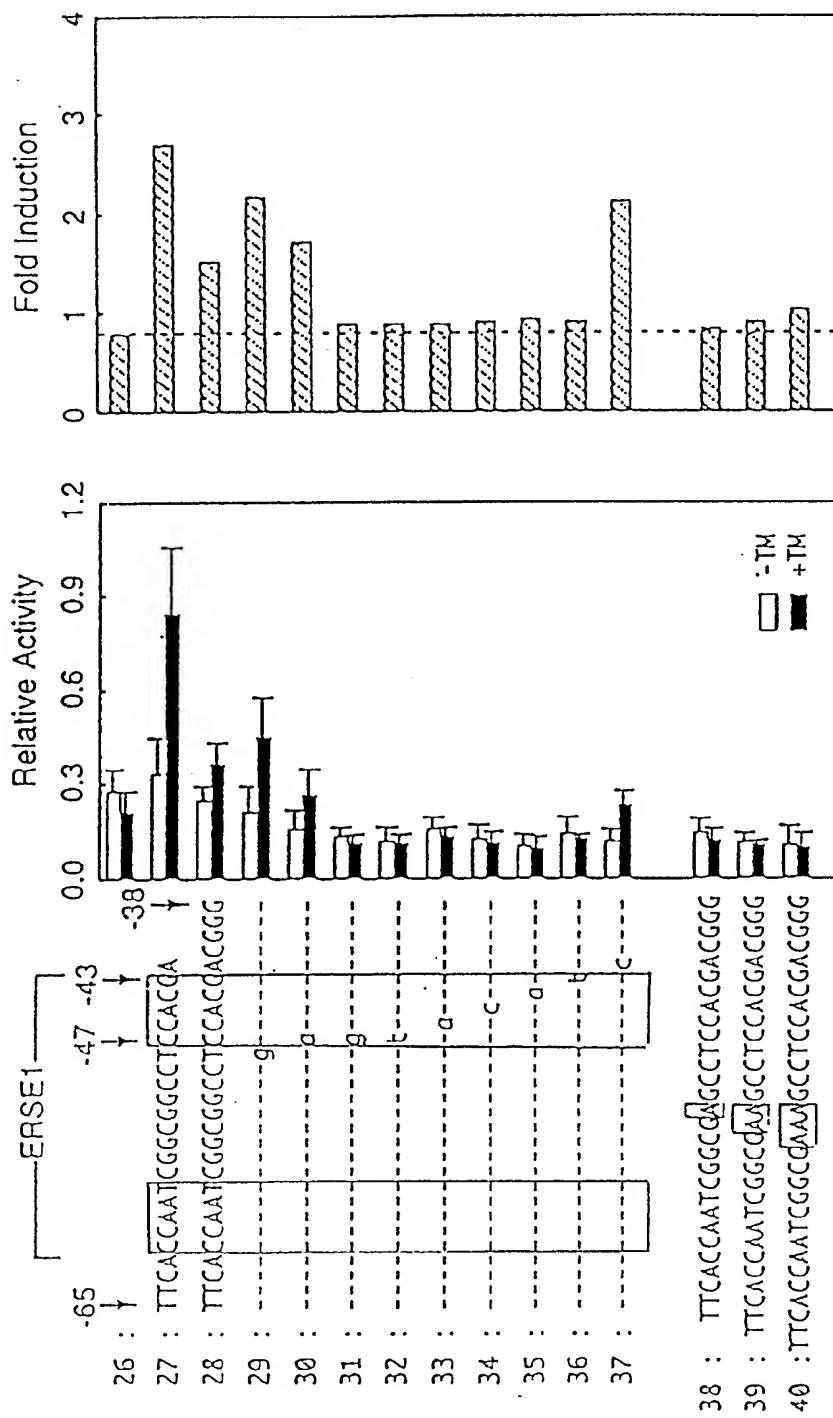
Dm	CRT	ERSE1		CCAATGAAAACGTTCCAGC
		ERSE2		CCAATCAGGGATGTCTACG
Ce	GRP78	ERSE1		CCAATCGGCGACGGCCGTG
	GRP78	ERSE2		CCTATCGTCCTAGGCCACG
At	GRP78	ERSE1		CCAATCAGGTTTTAACTCG
	CRT	ERSE1		CCAATAGGTAACCGACACG
	CRT	ERSE2		CCAATACTATAACGCCATG
So	GRP78	ERSE1		GGAATATCATTGGTCCACG
Rc	CRT	ERSE1	-185	CCAATCGTATTATGCCATG
	CRT	ERSE2	-243	CAAATACGATATTACCACG
	CRT	ERSE3	-314	CCCCTCATAGCACGCCACG
	CRT	ERSE4	-1695	CCATTCTTTGCTGCTCACG
An	GRP78	ERSE1		CCAATTGAGCAGCTCGTCG





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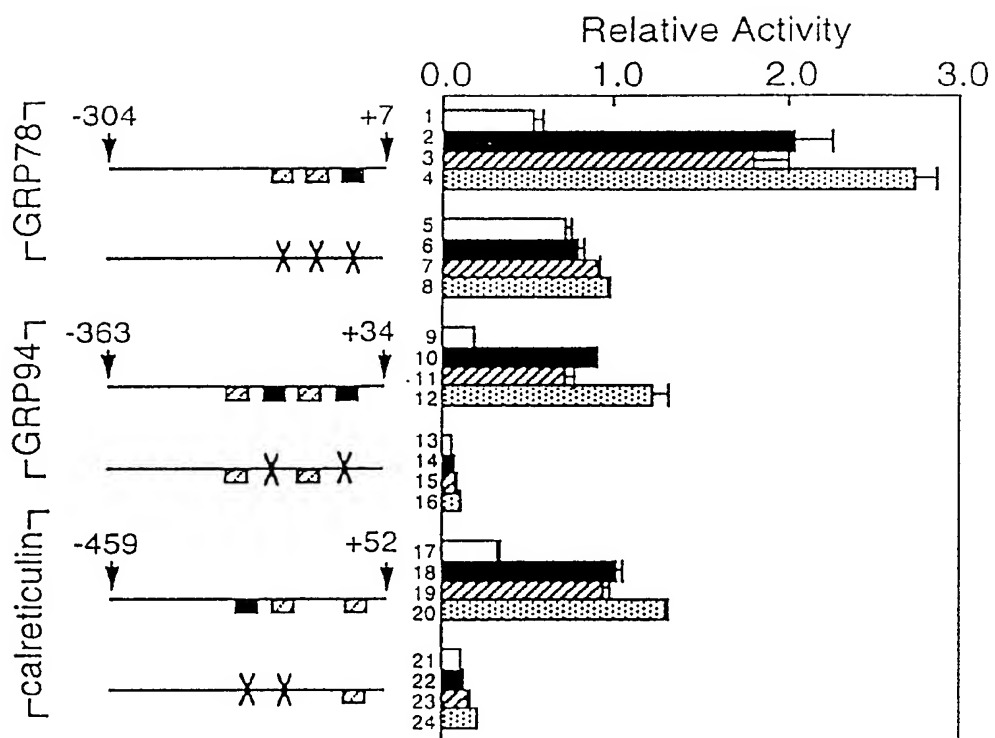


FIG. 10

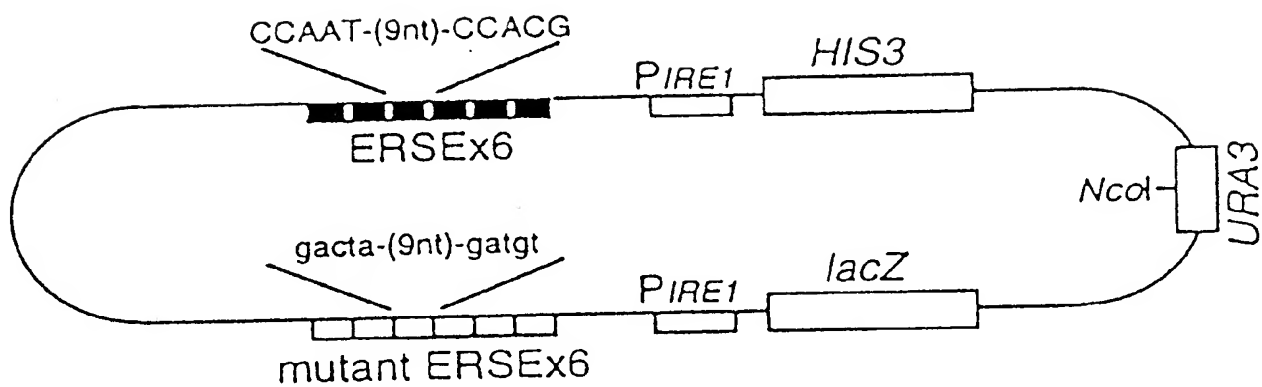
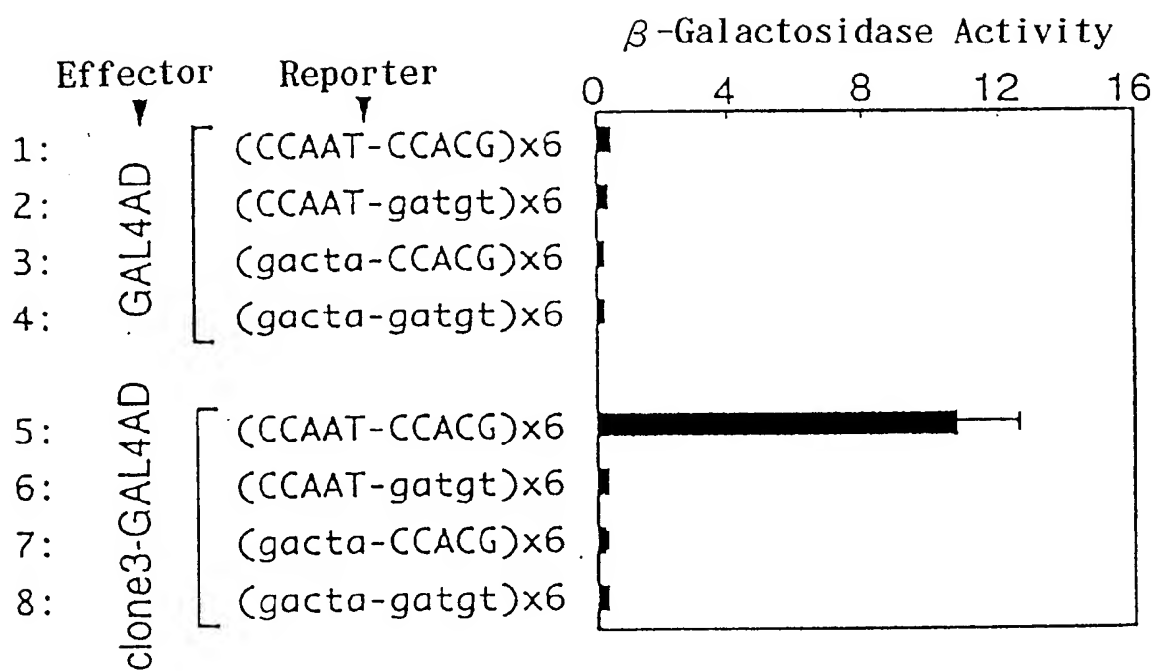


FIG. 11

1 2 / 3 2



F I G . 1 2

1 3 / 3 2

(A)

ATF6	RRQQRMIKNRESACQSRKKKKEY
Hac1	RRIERILRNRRAAHQSREKKRLH

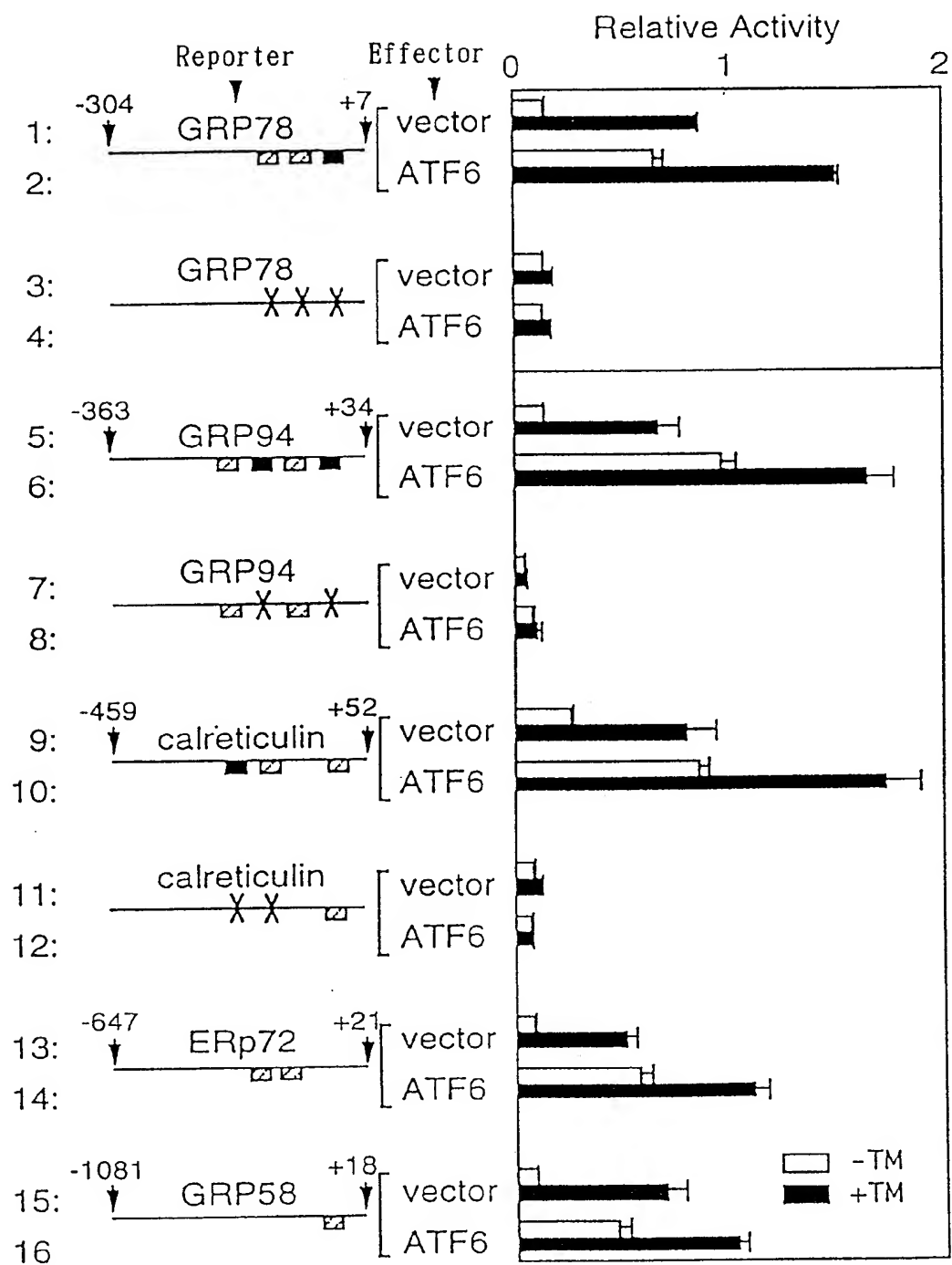
(B)

ATF6	RRQQRMIKNRESACQSRKKKKEY
CREB-RP/G13	KRQQRMIKNRESACQSRKKKKEY
CREB	KREVRLMKNREAARECRRKKKEY
ATF1	KREIRLMKNREAARECRRKKKEY
CRE-BP1/ATF2	KRRKFLERNRAAASRCRQKRKVW
ATF3	RKKRRRERNKIAAAKCRNKKKEK
ATF4	KKLKKMEQNKTAATRYRQKKRAE
c-fos(ATF5)	EKENPKERNKMAAAKCRNRRREL

(C)

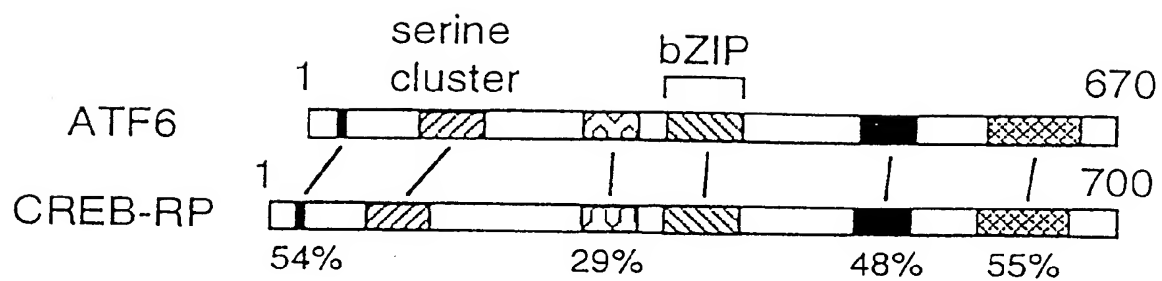
ATF6	RRQQRMIKNRESACQSRKKKKEY
GBF4	QRQQRMIKNRESAARSRERKQAY
DPBF1	RRQQRMIKNRESAARSRARKQAY
DPBF2	RRQQRMIKNRESAARSRARKQAY
C2546	RRQQRMIKNRESAARSRARKQAY

1 4 / 3 2



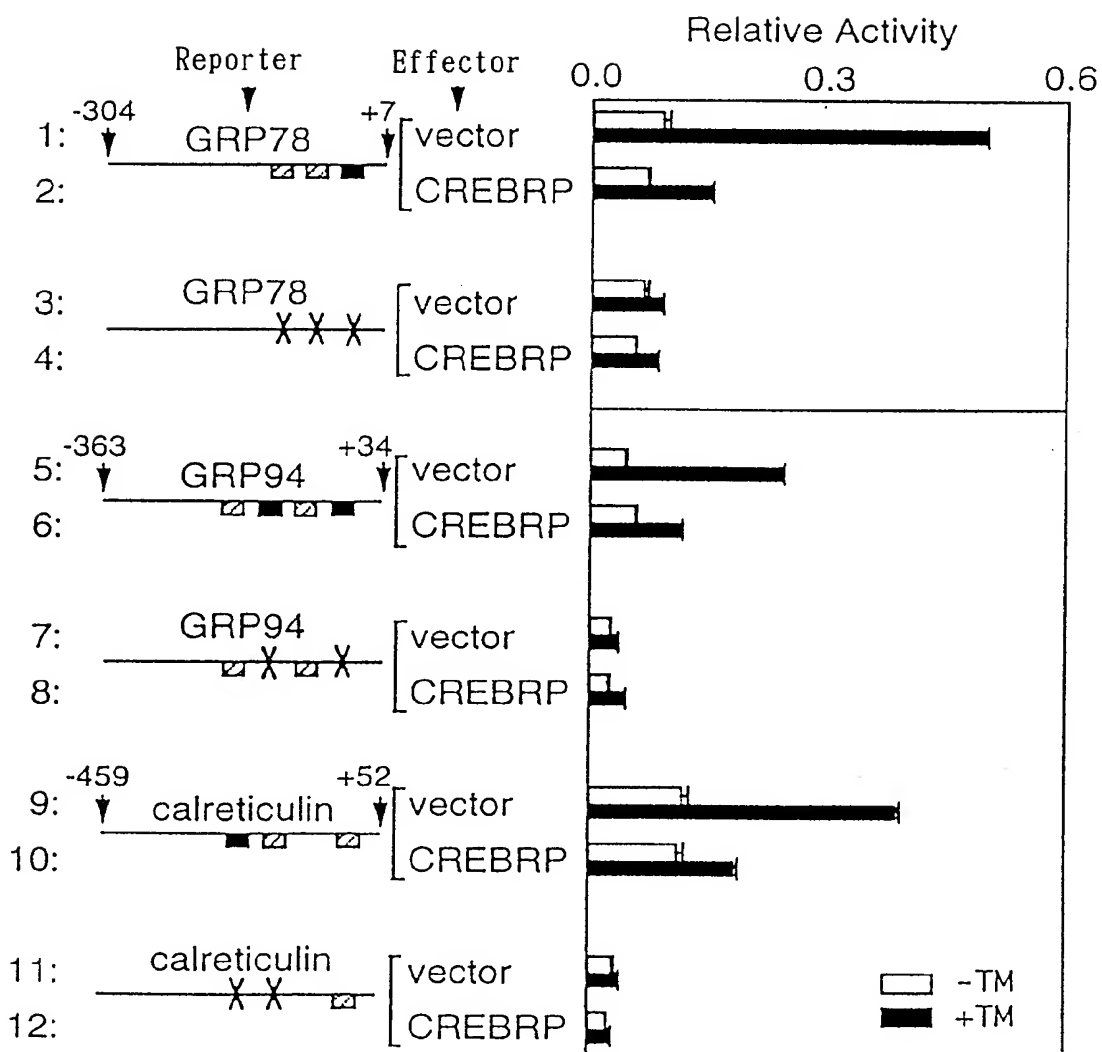
F I G . 1 4

1 5 / 3 2



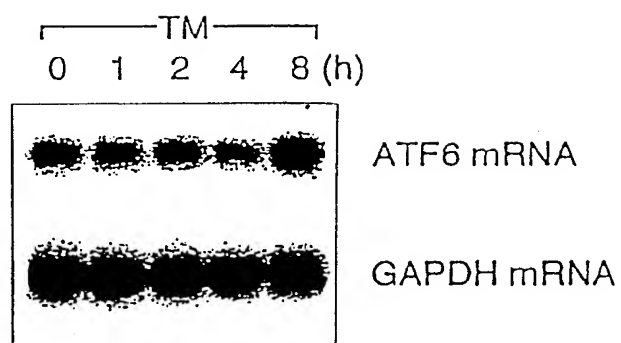
F I G . 1 5

1 6 / 3 2



F I G . 1 6

1 7 / 3 2



F I G . 1 7

18 / 32

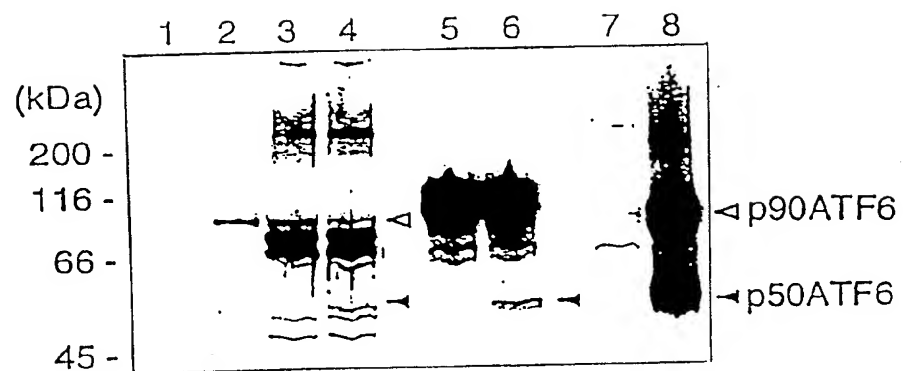


FIG. 18

19 / 32

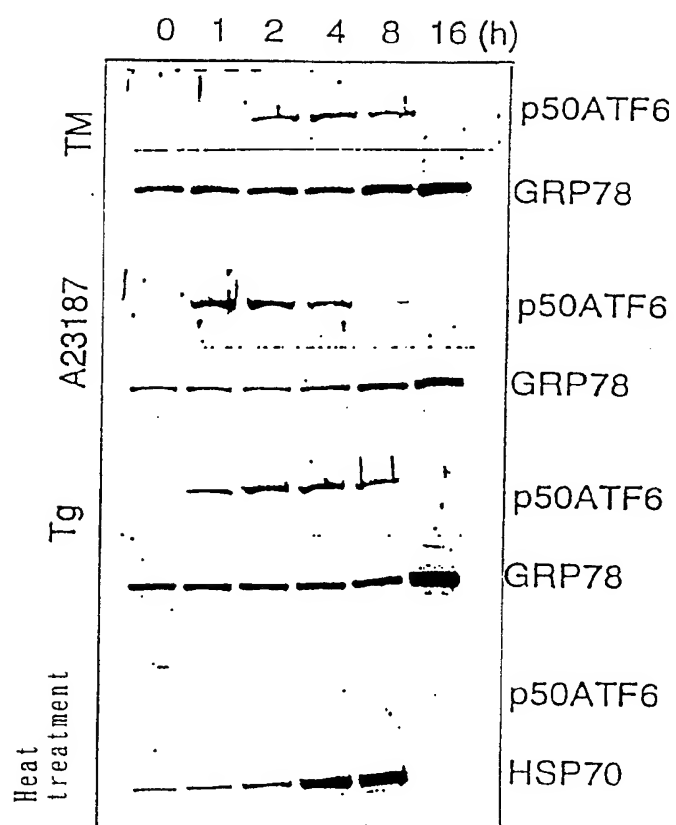
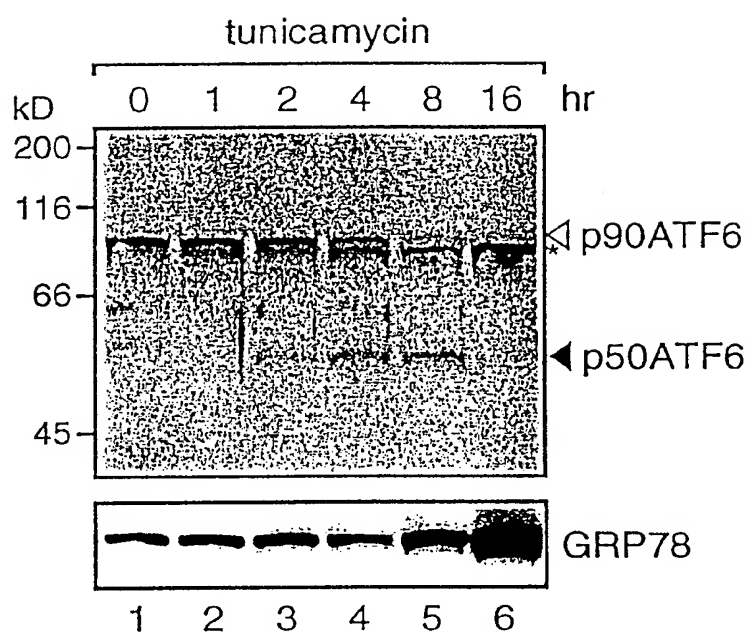


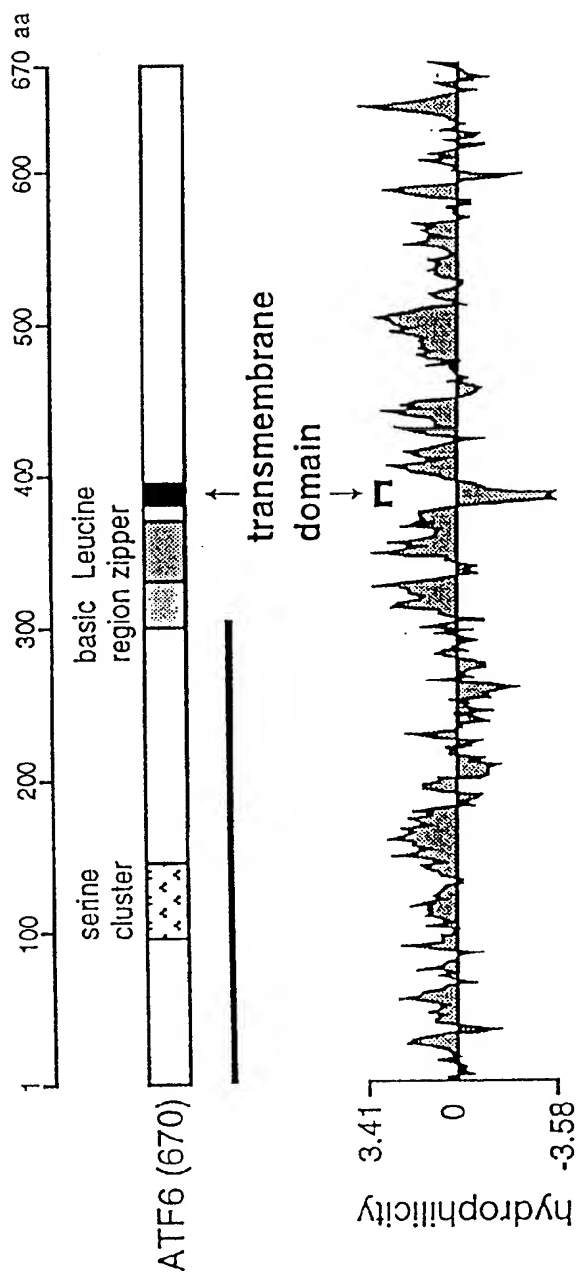
FIG. 19

20 / 32



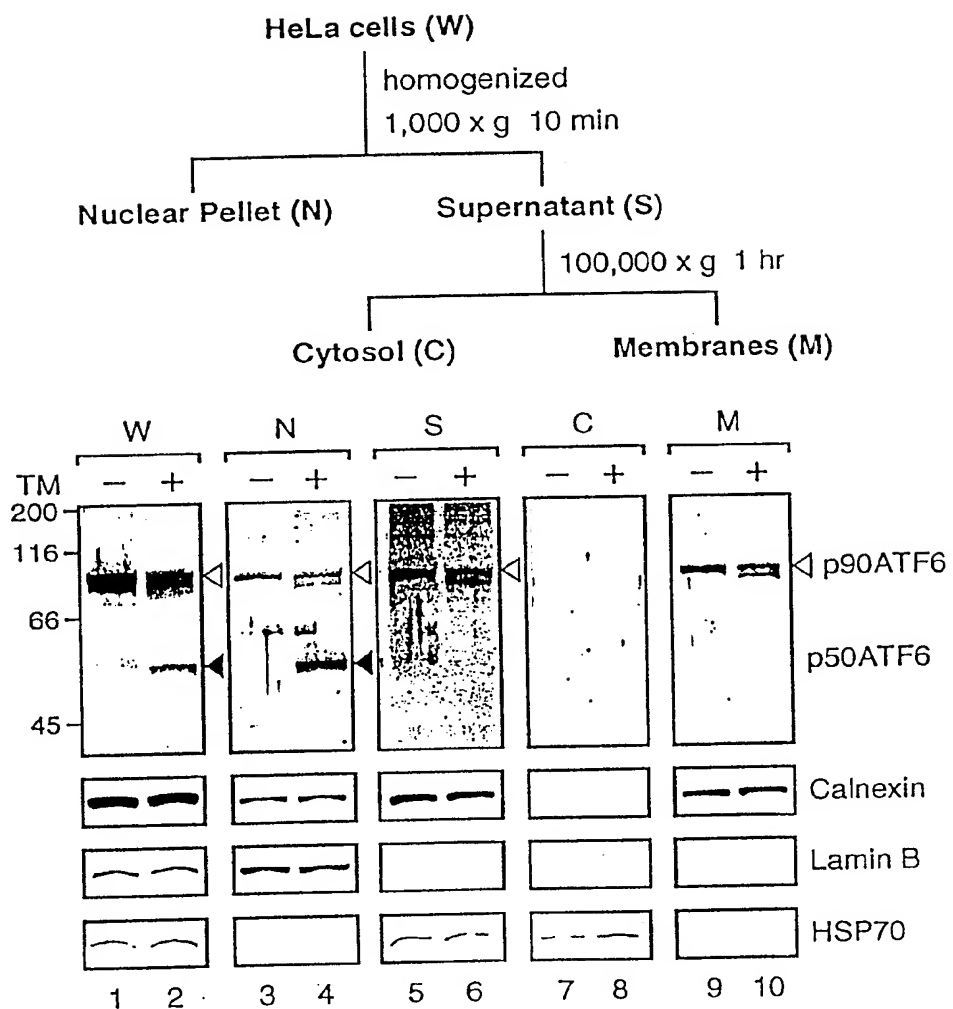
F I G . 2 0

2 1 / 3 2



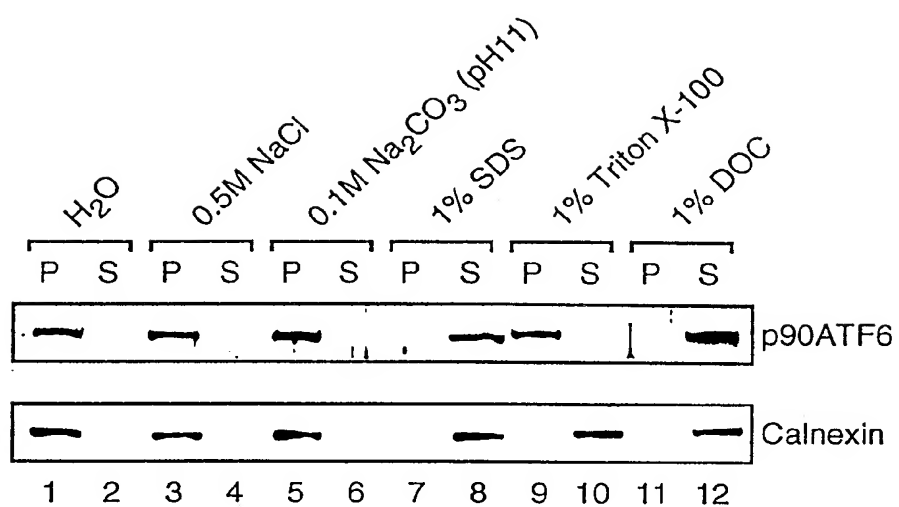
F I G . 2 1

2 2 / 3 2



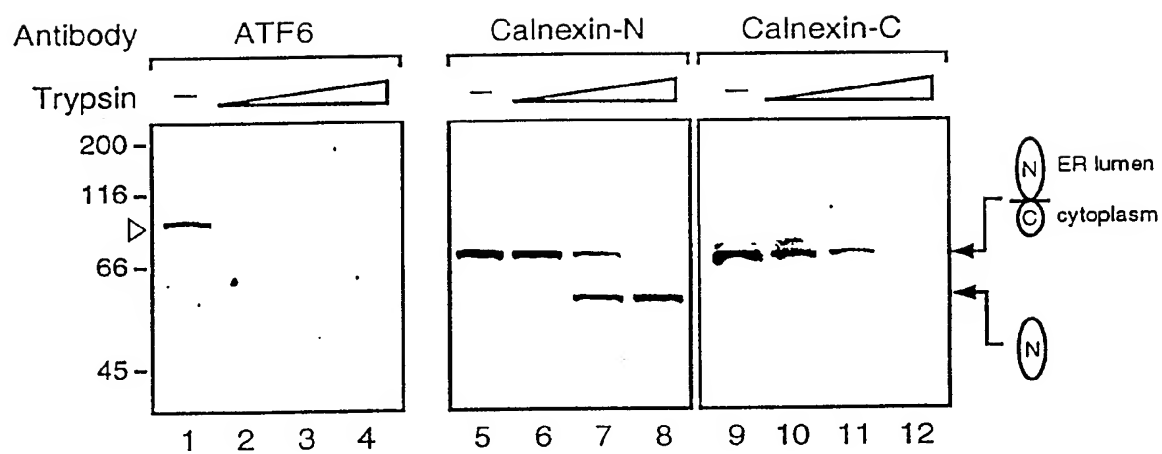
F I G . 2 2

2 3 / 3 2



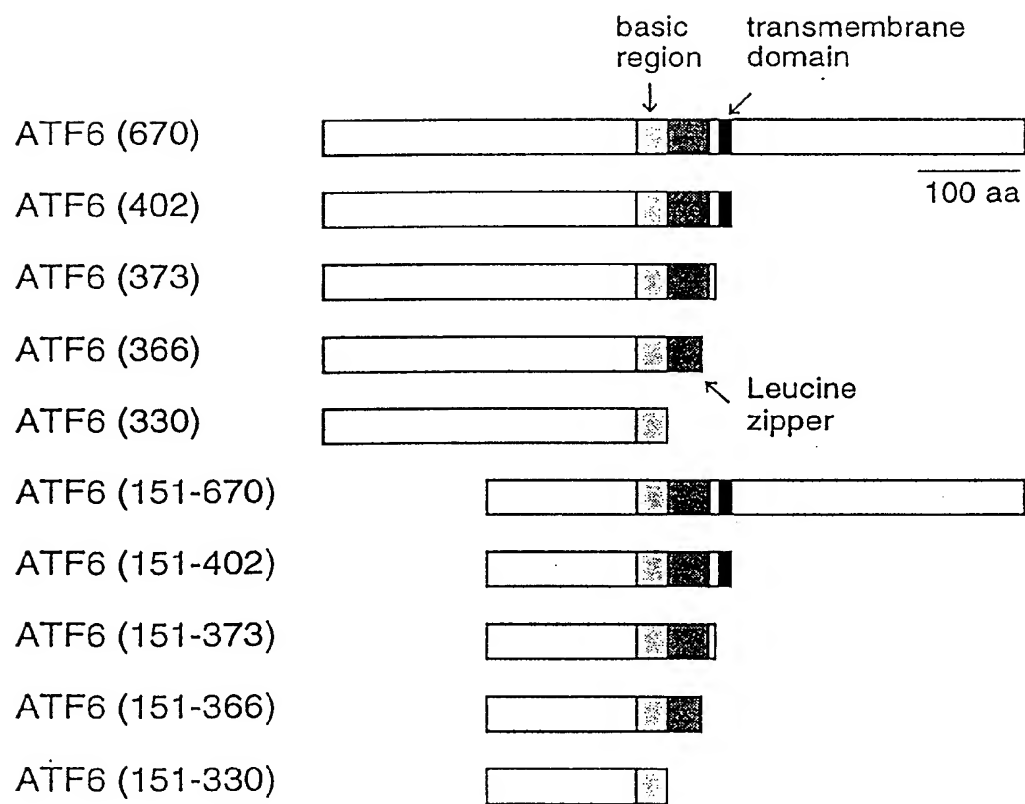
F I G . 2 3

2 4 / 3 2



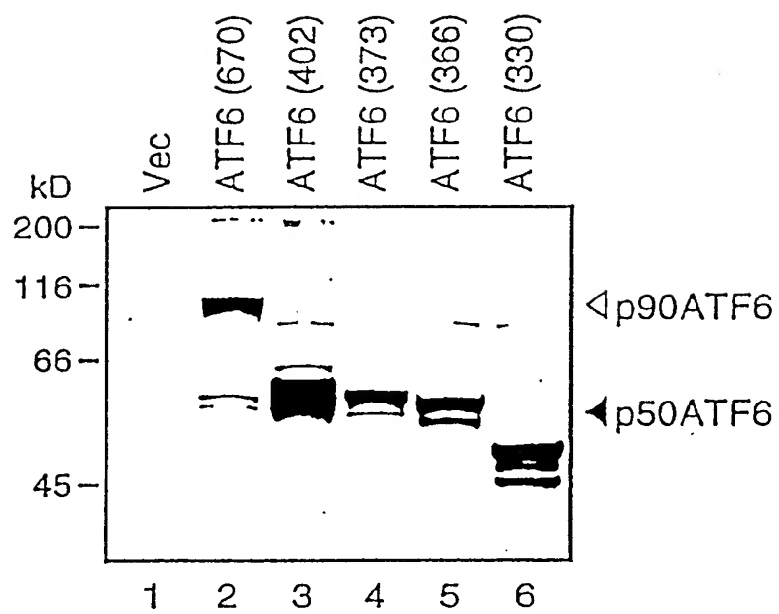
F I G . 2 4

2 5 / 3 2



F I G . 2 5

2 6 / 3 2



F I G . 2 6

27 / 32

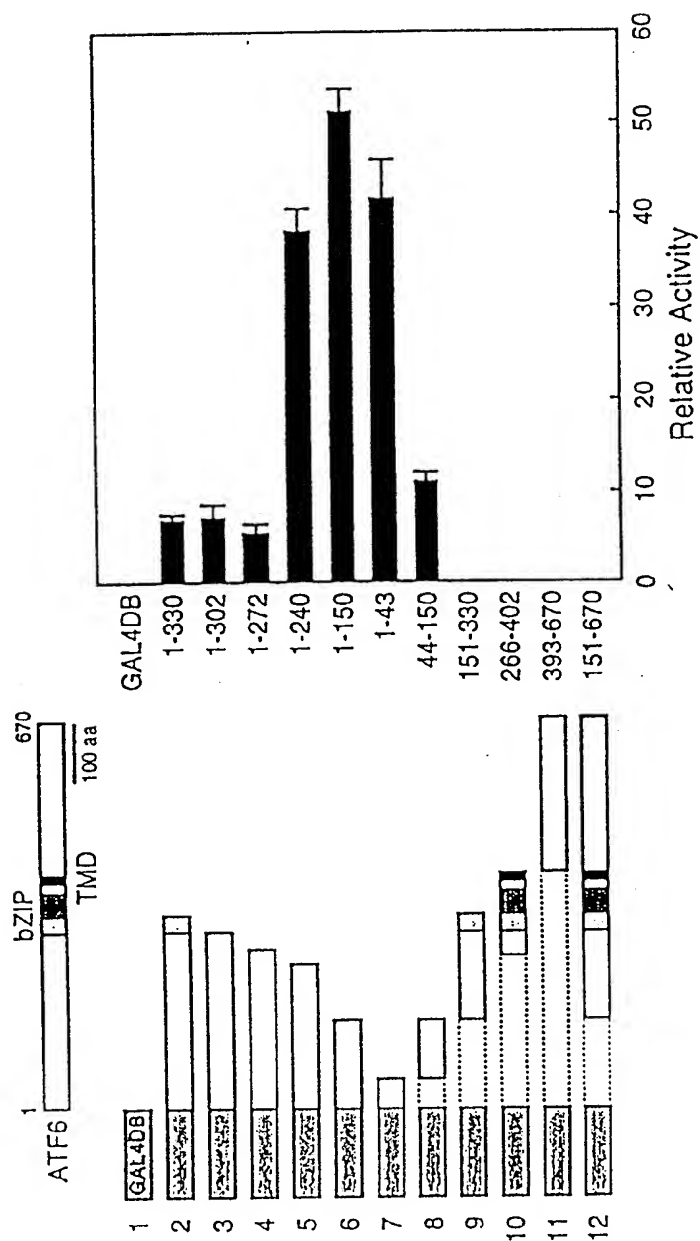


FIG. 27

28 / 32

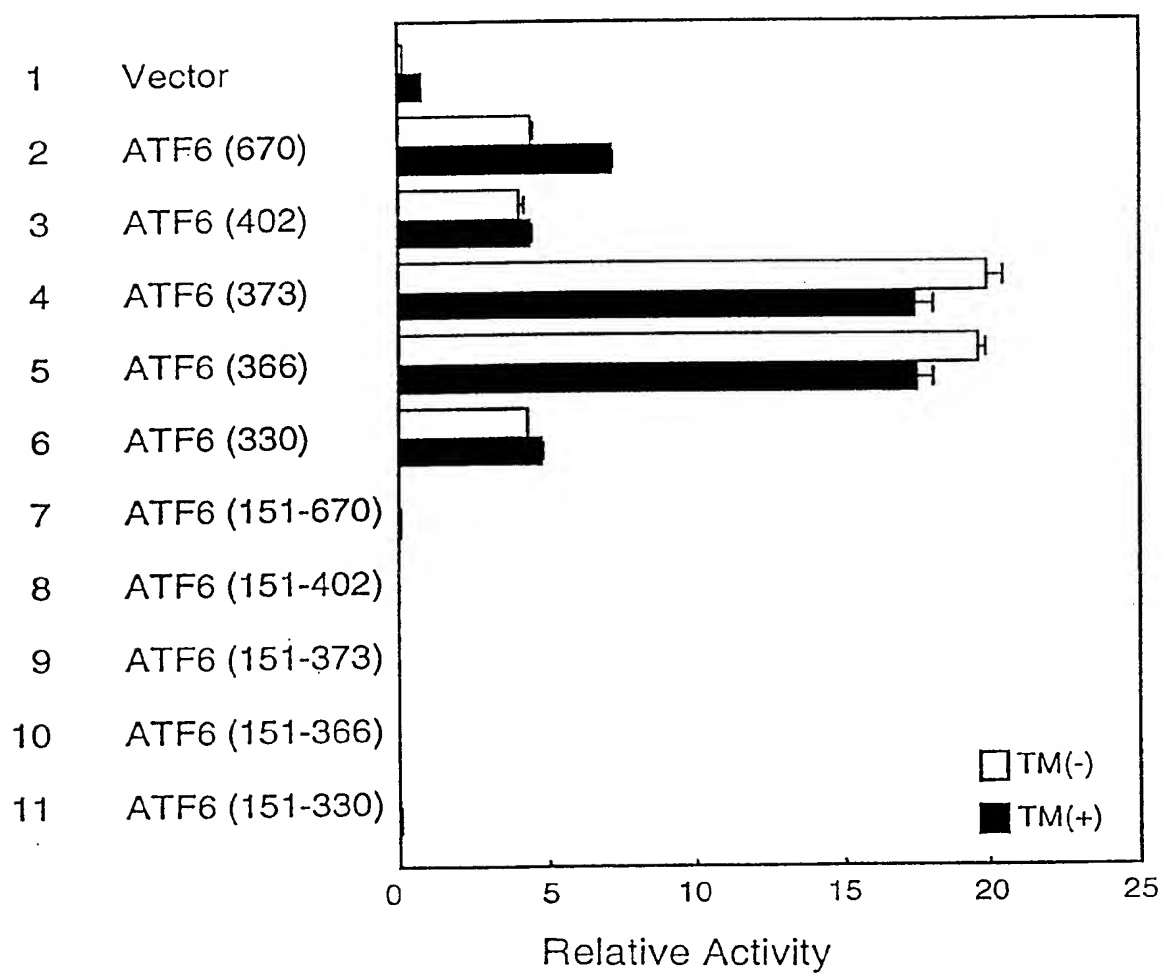
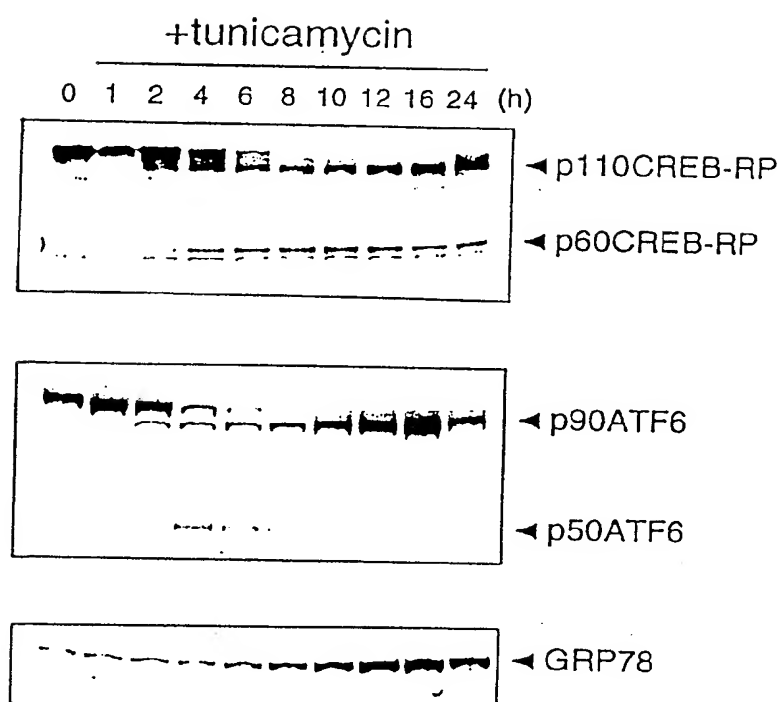


FIG. 28

2 9 / 3 2



F I G . 2 9

30 / 32

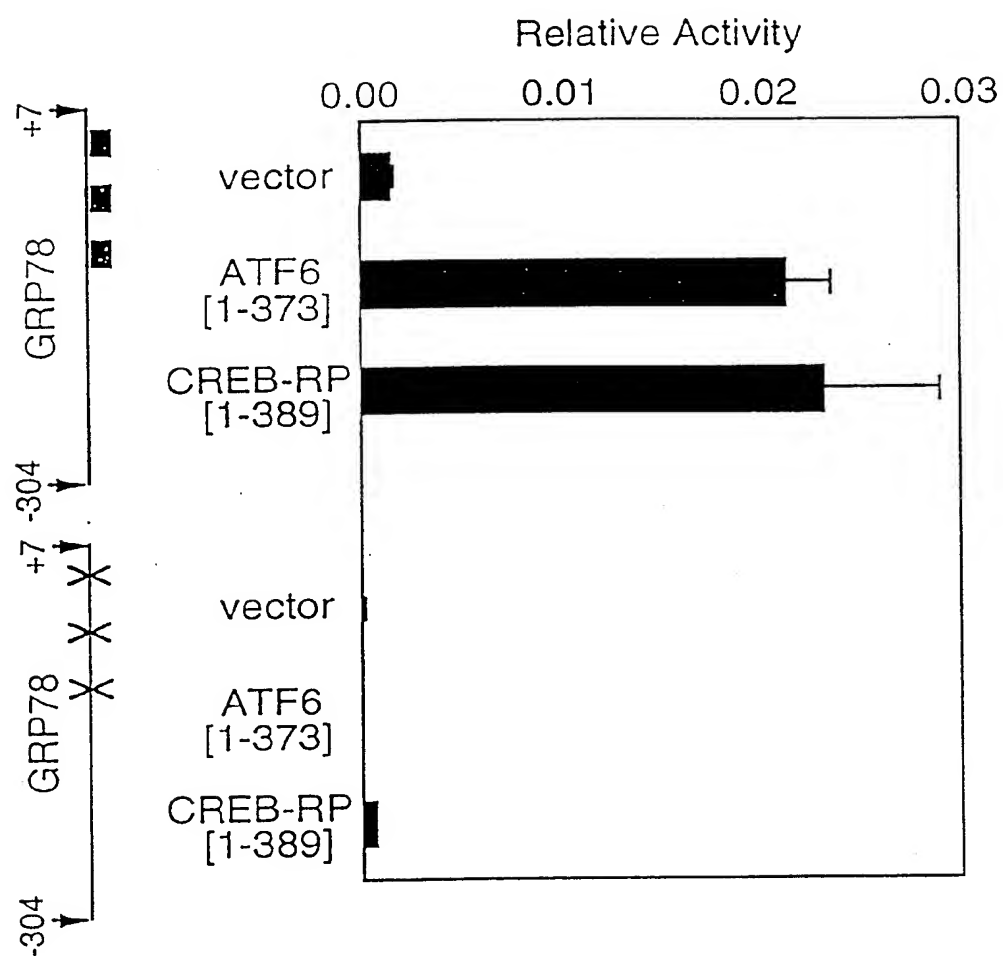
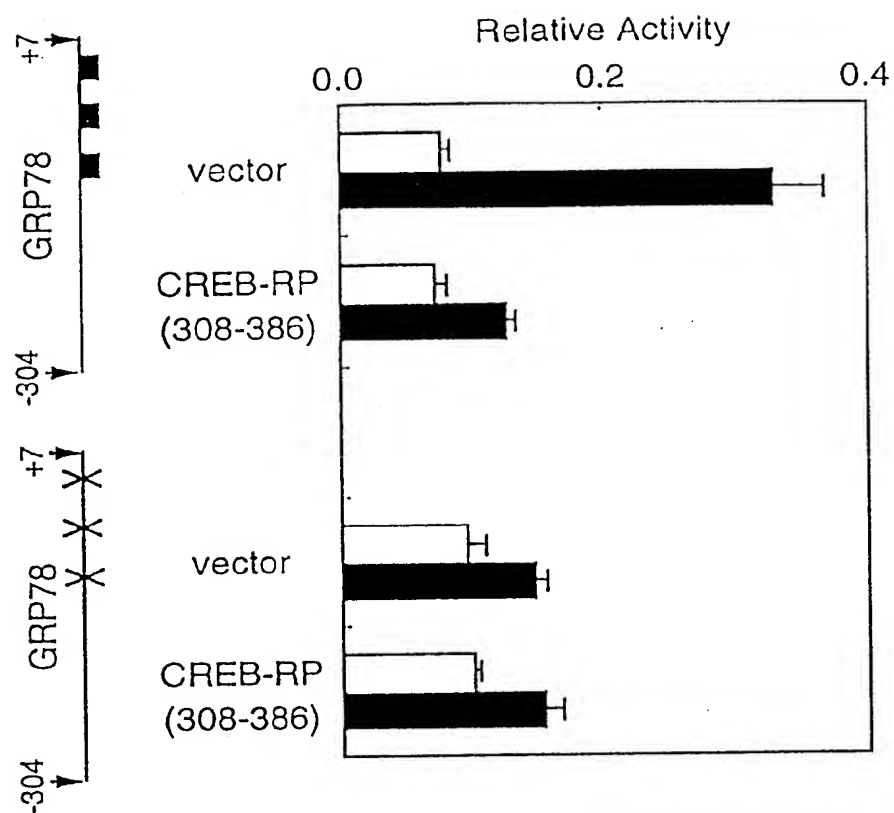


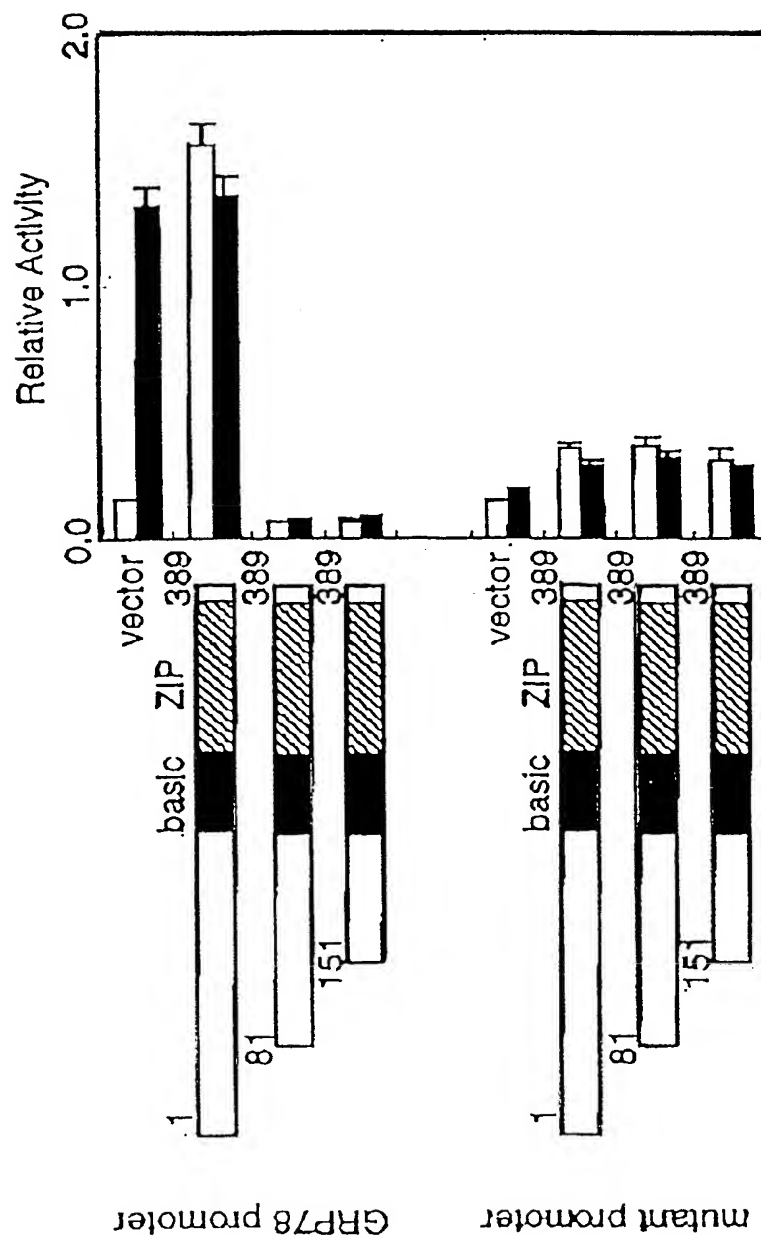
FIG. 30

3 1 / 3 2



F I G . 3 1

3 2 / 3 2



F I G . 3 2

SEQUENCE LISTING

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<120> Endoplasmic reticulum stress transcription factor

<130> 99-043-PCT

<150> JP 10-324227

<151> 1998-11-13

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<223> "n" is A or C or G or T.

<220>

<223> The sequence as shown in SEQ ID NO: 1 is the ERSE1 consensus seque

2 / 2 7

nce.

<400> 1

ccaatnnnnn nnnnccacg

19

<210> 2

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<220>

<223> The sequence as shown in SEQ ID NO: 2 is the ERSE2 consensus sequence.

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19

<210> 3

<211> 19

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<213> Artificial Sequence

3 / 2 7

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<222> 5, 6, 7, 8, 9, 10, 11, 12, 13, 14

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<220>

<223> The sequence as shown in SEQ ID NO: 3 is the ERSE3 consensus sequence.

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19

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<213> human

<400> 4

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aa

122

<210> 5

<211> 19

<212> DNA

<213> human

<400> 5

ccaatcggcg gcctccacg

19

<210> 6

<211> 19

<212> DNA

<213> murine

<400> 6

ccaatcggag gcctccacg

19

<210> 7

<211> 19

<212> DNA

<213> rat

<400> 7

ccaatcggag gcctccacg

19

<210> 8

<211> 19

<212> DNA

<213> human

<400> 8

ccaatcgcgc cgcaccacg

19

<210> 9

<211> 19

<212> DNA

<213> chicken

<400> 9

ccaatgggag cgcaccacg

19

<210> 10

<211> 19

<212> DNA

<213> human

<400> 10

ccaatcggaa ggagccacg

19

<210> 11

<211> 19

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<213> chicken

<400> 11

ccaatcgacg ccggccacg

19

<210> 12

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<213> human

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ccaatgatgg tcgaccacg

19

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<213> murine

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ccaatgaggg tcgaccacg

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ccaatgaacg gcctccaac

19

<210> 15

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<212> DNA

<213> murine

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ccaatcagcg gcctccaac

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<210> 24

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<400> 24

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19

1 0 / 2 7

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<211> 19

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<400> 27

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19

<210> 28

1 1 / 2 7

<211> 19

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<213> rat

<400> 28

ccagtcagaa tgcaacacg

19

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<212> DNA

1 2 / 2 7

<213> human

<400> 31

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caattcagcc caagccttta ttgcttccag cagcacccaa gactcaaaca aactccagt	660
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1 5 / 2 7

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<213> human

<400> 32

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35 40 45

Leu Glu Ala Ala Asn Glu Thr Tyr Glu Asn Asn Phe Asp Asn Leu

50 55 60

Asp Phe Asp Leu Asp Leu Met Pro Trp Glu Ser Asp Ile Trp Asp

65 70 75

Ile Asn Asn Gln Ile Cys Thr Val Lys Asp Ile Lys Ala Glu Pro

1 6 / 2 7

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Gln Pro Leu Ser	Pro Ala Ser Ser Ser	Tyr Ser Val Ser Ser	Pro		
	95		100		105
Arg Ser Val Asp	Ser Tyr Ser Ser Thr	Gln His Val Pro Glu Glu			
	110		115		120
Leu Asp Leu Ser	Ser Ser Ser Gln Met	Ser Pro Leu Ser Leu Tyr			
	125		130		135
Gly Glu Asn Ser	Asn Ser Leu Ser Ser	Pro Glu Pro Leu Lys Glu			
	140		145		150
Asp Lys Pro Val	Thr Gly Ser Arg Asn	Lys Thr Glu Asn Gly Leu			
	155		160		165
Thr Pro Lys Lys	Lys Ile Gln Val Asn	Ser Lys Pro Ser Ile Gln			
	170		175		180
Pro Lys Pro Leu	Leu Leu Pro Ala Ala	Pro Lys Thr Gln Thr Asn			
	185		190		195
Ser Ser Val Pro	Ala Lys Thr Ile Ile	Ile Gln Thr Val Pro Thr			
	200		205		210
Leu Met Pro Leu	Ala Lys Gln Gln Pro	Ile Ile Ser Leu Gln Pro			
	215		220		225
Ala Pro Thr Lys	Gly Gln Thr Val Leu	Leu Ser Gln Pro Thr Val			
	230		235		240
Val Gln Leu Gln	Ala Pro Gly Val Leu	Pro Ser Ala Gln Pro Val			
	245		250		255
Leu Ala Val Ala	Gly Gly Val Thr Gln	Leu Pro Asn His Val Val			
	260		265		270
Asn Val Val Pro	Ala Pro Ser Ala Asn	Ser Pro Val Asn Gly Lys			

1 7 / 2 7

275	280	285
Leu Ser Val Thr Lys Pro Val Leu Gln Ser Thr Met Arg Asn Val		
290	295	300
Gly Ser Asp Ile Ala Val Leu Arg Arg Gln Gln Arg Met Ile Lys		
305	310	315
Asn Arg Glu Ser Ala Cys Gln Ser Arg Lys Lys Lys Lys Glu Tyr		
320	325	330
Met Leu Gly Leu Glu Ala Arg Leu Lys Ala Ala Leu Ser Glu Asn		
335	340	345
Glu Gln Leu Lys Lys Glu Asn Gly Thr Leu Lys Arg Gln Leu Asp		
350	355	360
Glu Val Val Ser Glu Asn Gln Arg Leu Lys Val Pro Ser Pro Lys		
365	370	375
Arg Arg Val Val Cys Val Met Ile Val Leu Ala Phe Ile Ile Leu		
380	385	390
Asn Tyr Gly Pro Met Ser Met Leu Glu Gln Asp Ser Arg Arg Met		
395	400	405
Asn Pro Ser Val Ser Pro Ala Asn Gln Arg Arg His Leu Leu Gly		
410	415	420
Phe Ser Ala Lys Glu Ala Gln Asp Thr Ser Asp Gly Ile Ile Gln		
425	430	435
Lys Asn Ser Tyr Arg Tyr Asp His Ser Val Ser Asn Asp Lys Ala		
440	445	450
Leu Met Val Leu Thr Glu Glu Pro Leu Leu Tyr Ile Pro Pro Pro		
455	460	465
Pro Cys Gln Pro Leu Ile Asn Thr Thr Glu Ser Leu Arg Leu Asn		

1 8 / 2 7

470	475	480
His Glu Leu Arg Gly Trp Val His Arg His Glu Val Glu Arg Thr		
485	490	495
Lys Ser Arg Arg Met Thr Asn Asn Gln Gln Lys Thr Arg Ile Leu		
500	505	510
Gln Gly Ala Leu Glu Gln Gly Ser Asn Ser Gln Leu Met Ala Val		
515	520	525
Gln Tyr Thr Glu Thr Thr Ser Ser Ile Ser Arg Asn Ser Gly Ser		
530	535	540
Glu Leu Gln Val Tyr Tyr Ala Ser Pro Arg Ser Tyr Gln Asp Phe		
545	550	555
Phe Glu Ala Ile Arg Arg Arg Gly Asp Thr Phe Tyr Val Val Ser		
560	565	570
Phe Arg Arg Asp His Leu Leu Leu Pro Ala Thr Thr His Asn Lys		
575	580	585
Thr Thr Arg Pro Lys Met Ser Ile Val Leu Pro Ala Ile Asn Ile		
590	595	600
Asn Glu Asn Val Ile Asn Gly Gln Asp Tyr Glu Val Met Met Gln		
605	610	615
Ile Asp Cys Gln Val Met Asp Thr Arg Ile Leu His Ile Lys Ser		
620	625	630
Ser Ser Val Pro Pro Tyr Leu Arg Asp Gln Gln Arg Asn Gln Thr		
635	640	645
Asn Thr Phe Phe Gly Ser Pro Pro Ala Ala Thr Glu Ala Thr His		
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Val Val Ser Thr Ile Pro Glu Ser Leu Gln		

1 9 / 2 7

665

670

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cttccgttgc ccggagcagg atgtcccgtt tgacggcagc tccctggacg tggggatgga 240

tgtcagcccc tctgagcccc catgggaact cctgccgac ttcacagatc ttcaggtgaa 300

gtctgagcca tcttccccct gctcttctc ctcctcagc tccgagtcac cgcgtctctc 360

cacagagcca tccagcgagg ctcttggggg aggggaggtg ctccatgtga agacagagtc 420

cttggcacc ccaactgtgtc tcctgggaga tgaccaaca tcttcatttg aaaccgtcca 480

gatcaacgtt atccccacct ctgatgattc ctcagatgtc cagaccaaga tagaacctgt 540

2 0 / 2 7

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cctcctgtgg gatgtcccag cccctcact tggagctgtc cagatcagca tgggcccata 720

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cttcaacttt ggacctgtca gcatcagtga gcctccttca gctcccatct ctctcggat 1320

2 1 / 2 7

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gtccctgagg cttgctgacg agttgagtgg ctgggtccag cgccaccaga gaggccggag 1620

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gcaactatat cgccaccag accgttcgca gccagcattc ttgatgcaa ttgaccgacg 1800

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ccacaacaag acctcccggc ccaagatgtc cctggtgatg cctgccatgg ccccaatga 1920

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acagccatcc ccaaccccag gcaatgccac aggtggcccc ttgccagtct ctgcagccag 2100

2 2 / 2 7

ccaggccac caggcctccc accagcccct ctacctcaat catccctgac ctctgccatt 2160
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<211> 700

<212> PRT

<213> human

<400> 34

Met Ala Glu Leu Met Leu Leu Ser Glu Ile Ala Asp Pro Thr Arg

1

5

10

15

2 3 / 2 7

Phe	Phe	Thr	Asp	Asn	Leu	Leu	Ser	Pro	Glu	Asp	Trp	Asp	Ser	Thr
					20				25					30
Leu	Tyr	Ser	Gly	Leu	Asp	Glu	Val	Ala	Glu	Glu	Gln	Thr	Gln	Leu
					35				40					45
Phe	Arg	Cys	Pro	Glu	Gln	Asp	Val	Pro	Phe	Asp	Gly	Ser	Ser	Leu
					50				55					60
Asp	Val	Gly	Met	Asp	Val	Ser	Pro	Ser	Glu	Pro	Pro	Trp	Glu	Leu
					65				70					75
Leu	Pro	Ile	Phe	Pro	Asp	Leu	Gln	Val	Lys	Ser	Glu	Pro	Ser	Ser
					80				85					90
Pro	Cys	Ser	Ser	Ser	Ser	Leu	Ser	Ser	Glu	Ser	Ser	Arg	Leu	Ser
					95				100					105
Thr	Glu	Pro	Ser	Ser	Glu	Ala	Leu	Gly	Val	Gly	Glu	Val	Leu	His
					110				115					120
Val	Lys	Thr	Glu	Ser	Leu	Ala	Pro	Pro	Leu	Cys	Leu	Leu	Gly	Asp
					125				130					135
Asp	Pro	Thr	Ser	Ser	Phe	Glu	Thr	Val	Gln	Ile	Asn	Val	Ile	Pro
					140				145					150
Thr	Ser	Asp	Asp	Ser	Ser	Asp	Val	Gln	Thr	Lys	Ile	Glu	Pro	Val
					155				160					165
Ser	Pro	Cys	Ser	Ser	Val	Asn	Ser	Glu	Ala	Ser	Leu	Leu	Ser	Ala
					170				175					180
Asp	Ser	Ser	Ser	Gln	Ala	Phe	Ile	Gly	Glu	Glu	Val	Leu	Glu	Val
					185				190					195
Lys	Thr	Glu	Ser	Leu	Ser	Pro	Ser	Gly	Cys	Leu	Leu	Trp	Asp	Val
					200				205					210

2 4 / 2 7

Pro Ala Pro Ser Leu Gly Ala Val Gln Ile Ser Met Gly Pro Ser		
	215	220 225
Leu Asp Gly Ser Ser Gly Lys Ala Leu Pro Thr Arg Lys Pro Pro		
	230	235 240
Leu Gln Pro Lys Pro Val Val Leu Thr Thr Val Pro Met Pro Ser		
	245	250 255
Arg Ala Val Pro Pro Ser Thr Thr Val Leu Leu Gln Ser Leu Val		
	260	265 270
Gln Pro Pro Pro Val Ser Pro Val Val Leu Ile Gln Gly Ala Ile		
	275	280 285
Arg Val Gln Pro Glu Gly Pro Ala Pro Ser Leu Pro Arg Pro Glu		
	290	295 300
Arg Lys Ser Ile Val Pro Ala Pro Met Pro Gly Asn Ser Cys Pro		
	305	310 315
Pro Glu Val Asp Ala Lys Leu Leu Lys Arg Gln Gln Arg Met Ile		
	320	325 330
Lys Asn Arg Glu Ser Ala Cys Gln Ser Arg Arg Lys Lys Lys Glu		
	335	340 345
Tyr Leu Gln Gly Leu Glu Ala Arg Leu Gln Ala Val Leu Ala Asp		
	350	355 360
Asn Gln Gln Leu Arg Arg Glu Asn Ala Ala Leu Arg Arg Arg Leu		
	365	370 375
Glu Ala Leu Leu Ala Glu Asn Ser Glu Leu Lys Leu Gly Ser Gly		
	380	385 390
Asn Arg Lys Val Val Cys Ile Met Val Phe Leu Leu Phe Ile Ala		
	395	400 405

2 5 / 2 7

Phe Asn Phe Gly Pro Val Ser Ile Ser Glu Pro Pro Ser Ala Pro		
410	415	420
Ile Ser Pro Arg Met Asn Lys Gly Glu Pro Gln Pro Arg Arg His		
425	430	435
Leu Leu Gly Phe Ser Glu Gln Glu Pro Val Gln Gly Val Glu Pro		
440	445	450
Leu Gln Gly Ser Ser Gln Gly Pro Lys Glu Pro Gln Pro Ser Pro		
455	460	465
Thr Asp Gln Pro Ser Phe Ser Asn Leu Thr Ala Phe Pro Gly Gly		
470	475	480
Ala Lys Glu Leu Leu Leu Arg Asp Leu Asp Gln Leu Phe Leu Ser		
485	490	495
Ser Asp Cys Arg His Phe Asn Arg Thr Glu Ser Leu Arg Leu Ala		
500	505	510
Asp Glu Leu Ser Gly Trp Val Gln Arg His Gln Arg Gly Arg Arg		
515	520	525
Lys Ile Pro Gln Arg Ala Gln Glu Arg Gln Lys Ser Gln Pro Arg		
530	535	540
Lys Lys Ser Pro Pro Val Lys Ala Val Pro Ile Gln Pro Pro Gly		
545	550	555
Pro Pro Glu Arg Asp Ser Val Gly Gln Leu Gln Leu Tyr Arg His		
560	565	570
Pro Asp Arg Ser Gln Pro Ala Phe Leu Asp Ala Ile Asp Arg Arg		
575	580	585
Glu Asp Thr Phe Tyr Val Val Ser Phe Arg Arg Gly His Leu Leu		
590	595	600

2 6 / 2 7

Leu Pro Ala Ile Ser His Asn Lys Thr Ser Arg Pro Lys Met Ser
 605 610 615
 Leu Val Met Pro Ala Met Ala Pro Asn Glu Thr Leu Ser Gly Arg
 620 625 630
 Gly Ala Pro Gly Asp Tyr Glu Glu Met Met Gln Ile Glu Cys Glu
 635 640 645
 Val Met Asp Thr Arg Val Ile His Ile Lys Thr Ser Thr Val Pro
 650 655 660
 Pro Ser Leu Arg Lys Gln Pro Ser Pro Thr Pro Gly Asn Ala Thr
 665 670 675
 Gly Gly Pro Leu Pro Val Ser Ala Ala Ser Gln Ala His Gln Ala
 680 685 690
 Ser His Gln Pro Leu Tyr Leu Asn His Pro
 695 700

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<211> 29

<212> DNA

<213> Artificial Sequence

<400> 35

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29

<210> 36

<211> 29

<212> DNA

2 7 / 2 7

<213> human

<400> 36

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29